June 2020

therascreen® EGFR RGQ PCR Kit Instructions for Use (Handbook)

Σ 24

Version 1

IVD

For in vitro diagnostic use

For prescription use only

For use with Rotor-Gene® Q MDx instrument

For use with QIAamp® DSP DNA FFPE Tissue Kit

REF 870121

QIAGEN GmbH, QIAGEN Strasse 1,
40724, Hilden, GERMANY

R10 MAT 1121933EN

Contents

Intended Use

The *therascreen*® EGFR RGQ PCR Kit is a real-time PCR test for the qualitative detection of defined mutations of the epidermal growth factor receptor (EGFR) gene in DNA derived from formalin-fixed paraffin-embedded (FFPE) tumor tissue from non-small cell lung cancer (NSCLC) patients.

The test is intended to aid in identifying patients with NSCLC whose tumors have defined EGFR mutations and for whom safety and efficacy of a drug have been established as follows:

GILOTRIF® (afatinib)	Exon 19 deletions, L858R, L861Q, G719X, and S768I
IRESSA® (gefitinib) and VIZIMPRO® (dacomitinib)	Exon 19 deletions and L858R

Drug safety and efficacy have not been established for the following EGFR mutations also detected by the *therascreen*® EGFR RGQ PCR Kit:

GILOTRIF® (afatinib)	T790M and Exon 20 Insertions
IRESSA® (gefitinib) and VIZIMPRO® (dacomitinib)	T790M, L861Q, G719X, S768I and Exon 20 Insertions

Specimens are processed using the QIAamp DSP DNA FFPE Tissue Kit for manual sample preparation and the Rotor-Gene Q MDx instrument for automated amplification and detection.

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) cancer therapies in patients with 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 real-time qualitative PCR assay used on the Rotor-Gene Q MDx instrument. Using Scorpions® (10) and ARMS (Allele Refractory Mutation System) technologies (11), the *therascreen* EGFR RGQ PCR Kit enables the detection of 20 (see note [**] under Table 1) mutations in exons 18, 19, 20, and 21 of the EGFR oncogene against a background of wild type genomic DNA.

Among the 20 (see note [**] under Table 1) EGFR mutations detected by the *therascreen* EGFR RGQ PCR Kit, safety and efficacy of GILOTRIF has been established for the 17 (see note [**] under Table 1) mutations listed in Table 1, but has not been established for the 3 mutations listed in Table 2. Refer to GILOTRIF drug labeling for more details.

Table 1. List of mutations and COSMIC identities – safety and efficacy of GILOTRIF established

Mutation	Exon	Base change	COSMIC ID*
G719A	18	2156G>C	6239
Deletions	19	2238_2255del18	6220
		2235_2249del15	6223
		2236_2250del15	6225
		2239_2253del15	6254**
		2239_2256del18	6255
		2240_2254del15	12369**
		2240_2257del18	12370
		2239_2248TTAAGAGAAG>C	12382
		2239_2251>C	12383
		2237_2255>T	12384
		2239_2258>CA	12387
		2238_2252>GCA	12419
		2238_2248>GC	12422
		2235_2252>AAT	13551
S768I	20	2303G>T	6241
L858R	21	2573T>G	6224
L861Q	21	2582T>A	6213

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

** The COSM6254 (2239_2253del15) and COSM12369 (2240_2254del15) mutations result in the deletion of 15 base pairs from the EGFR sequence. The same final sequence is generated by both mutations and these mutations are indistinguishable from one another. Therefore, the mutation COSM6254 (2239_2253del15) has been removed from the most recent version of COSMIC (v83) and both mutations are now represented by COSM12369 (2240_2254del15). This follows the HGVS guideline to represent the most 3′ deletion. The *therascreen* EGFR test does not distinguish between any of the 19 deletion mutations and any positive deletion is called "Deletions". This change affects documentation only and does not affect the kit or its ability to detect any individual mutation.

Table 2. List of mutations and COSMIC identities – safety and efficacy of GILOTRIF not established

Mutation	Exon	Base change	COSMIC ID*
T790M	20	2369C>T	6240
Insertions	20	2319_2320insCAC	12377
		2310_2311insGGT	12378

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

Among the 20 (see note [**] under Table 1) EGFR mutations detected by the *therascreen* EGFR RGQ PCR Kit, safety and efficacy of IRESSA (gefitinib) and VIZIMPRO (dacomitinib) has been established for the 14 [see note [**] under Table 1) mutations listed in Table 3, but has not been established for the 6 mutations listed in Table 4. Refer to IRESSA (gefitinib) and VIZIMPRO (dacomitinib) drug labeling for more details.

Table 3. List of mutations and COSMIC identities – safety and efficacy of IRESSA and VIZIMPRO established

Mutation	Exon	Base change	COSMIC ID*
Deletions	19	2238_2255del18	6220
		2235_2249del15	6223
		2236_2250del15	6225
		2239_2253del15	6254**
		2239_2256del18	6255
		2240_2254del15	12369**
		2240_2257del18	12370
		2239_2248TTAAGAGAAG>C	12382
		2239_2251>C	12383
		2237_2255>T	12384
		2239_2258>CA	12387
		2238_2252>GCA	12419
		2238_2248>GC	12422
		2235_2252>AAT	13551
L858R	21	2573T>G	6224

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

** The COSM6254 (2239_2253del15) and COSM12369 (2240_2254del15) mutations result in the deletion of 15 base pairs from the EGFR sequence. The same final sequence is generated by both mutations and these mutations are indistinguishable from one another. Therefore, the mutation COSM6254 (2239_2253del15) has been removed from the most recent version of COSMIC (v83) and both mutations are now represented by COSM12369 (2240_2254del15). This follows the HGVS guideline to represent the most 3′ deletion. The *therascreen* EGFR test does not distinguish between any of the 19 deletion mutations and any positive deletion is called "Deletions". This change affects documentation only and does not affect the kit or its ability to detect any individual mutation.

Table 4. List of mutations and COSMIC identities – safety and efficacy of IRESSA and VIZIMPRO not established

Mutation	Exon	Base change	COSMIC ID*
T790M	20	2369C>T	6240
L861Q	21	2582T>A	6213
G719A	18	2156G>C	6239
S768I	20	2303G>T	6241
Insertions	20	2319_2320insCAC	12377
		2310_2311insGGT	12378

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

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 principal components of the kit are explained below.

Mutation Reaction Mixes

Each mutation-specific reaction mix uses mutation-specific ARMS primers to selectively amplify mutated DNA and a Scorpions primer to detect the amplification product.

ARMS

Allele-specific amplification is achieved by ARMS, which exploits the ability of *Taq* DNA polymerase to distinguish between a matched and a mismatched base at the 3′ end of a PCR primer. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3′ base is mismatched, only low-level background amplification may occur. Therefore, a mutated sequence is selectively amplified even in samples where the majority of the DNA does not carry the mutation.

Scorpions

Detection of amplification is performed using Scorpions. Scorpions are bifunctional molecules containing a PCR primer covalently linked to a probe. The probe incorporates the fluorophore carboxyfluorescein (FAM™) and a quencher. The latter quenches the fluorescence of the fluorophore. When the probe binds to the ARMS amplicon during PCR, the fluorophore and quencher become separated, leading to a detectable increase in fluorescence.

Control Reaction

The control reaction mix (tube CTRL) uses a Scorpions primer and an unlabeled primer to amplify a short sequence of exon 2 of the EGFR gene. The control reaction is used to determine

if an appropriate level of amplifiable DNA is present in the sample and is a factor in the analytical calculations that determine mutation status.

Internal Control

Each of the 8 reaction mixes contains an internal control designed to detect failure of the reaction (e.g., due to the presence of inhibitors). The internal control employs a non-EGFR related oligonucleotide target sequence, an unlabeled primer, and a Scorpions primer labeled with hexachlorofluorescein (HEX™) in order to distinguish it from the FAM-labeled Scorpions in the control and mutation reaction mixes.

Positive Control

The positive control (tube PC) comprises a mixture of synthetic oligonucleotides representing one mutation for each of the mutation assays and the control assay. Detection of the mutations within acceptable ranges confirms the proper functioning of each of the reaction mixes in the kit.

Negative Control

The no template control (tube NTC) contains nuclease-free water to be used for the "no template control" (NTC) reaction. The NTC serves as a negative control and assesses potential contamination during assay setup.

Sample Diluent

The sample diluent (tube Dil.) contains nuclease-free water.

Taq DNA Polymerase

Taq DNA polymerase is the enzyme for the polymerase chain reaction used by the *therascreen* EGFR RGQ PCR Kit.

Platform and Software

- The *therascreen* EGFR RGQ PCR Kit is specifically designed for use with the Rotor-Gene Q MDx instrument installed with the Rotor-Gene Q *therascreen* EGFR Assay Package version 3.1.2, available for download in the *therascreen* EGFR RGQ PCR Kit product webpage at **www.qiagen.com**. Go to **Product Resources** > **Supplementary Protocols** to download the assay package. Refer to the instrument user manual for information concerning the instrument.
- Refer to "Appendix: Installation of the Rotor-Gene Q *therascreen* EGFR Assay Package," for instructions on installing the Rotor-Gene Q *therascreen* EGFR Assay Package.

The Rotor-Gene Q MDx instrument must be maintained according to the requirements in the instrument user manual.

The Rotor-Gene Q MDx instrument is programmed for different cycle parameters, or "runs," by the Rotor-Gene Q *therascreen* EGFR Assay Package.

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

The HEX signal is read by the yellow channel in the Rotor-Gene Q MDx instrument, and the FAM signal is read by the green channel.

Materials Provided

Kit contents

***therascreen* EGFR RGQ PCR Kit**			
Catalog no.			**870121**
Number of reactions			**24**
Color	**Identity**	**Tube identification**	**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 Instructions for Use (Handbook)*		1

Reagents

Reaction mixes are duplex, containing FAM-labeled reagents to detect targets and a HEX-labeled internal control. Refer to Table 5.

Table 5. Reagents supplied in the *therascreen* EGFR RGQ PCR Kit

Reagent	Components	Volume
Control Reaction Mix	Control Unlabeled Primer, Control Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	2 tubes x 600 µl
T790M Reaction Mix	T790M ARMS Primers, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-Free Water	1 tube x 600 µl
Deletions Reaction Mix	Deletions ARMS Primers, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	1 tube x 600 µl
L858R Reaction Mix	L858R ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	1 tube x 600 µl
L861Q Reaction Mix	L861Q ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	1 tube x 600 µl
G719X Reaction Mix	G719X ARMS Primers, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	1 tube x 600 µl

Table continued on next page

Table continued from previous page

Reagent	Components	Volume
S768I Reaction Mix	S768I ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	1 tube x 600 µl
Insertions Reaction Mix	Insertions ARMS Primers, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpions Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer, Nuclease-free Water	1 tube x 600 µl
EGFR Positive Control	Control Template, T790M Template, Deletion 6223 Template, L858R Template, L861Q Template, G719A Template, S768I Template, Insertion 12378 Template, Poly A RNA, Tris EDTA Buffer	1 tube x 300 µl
Taq DNA Polymerase	*Taq* Polymerase: 50% Glycerol/Nuclease-free water	2 tubes x 80 µl
No-template control	Nuclease-Free Water	1 tube x 1.9 ml
For sample dilution	Nuclease-Free Water	1 tube x 1.9 ml

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

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404; see "DNA ", page 24)

Consumables

- 0.1 ml Strip Tubes and Caps, for use with 72-well rotor (QIAGEN, cat. no. 981103 or 981106)
- Nuclease-free, low DNA-binding microcentrifuge tubes for preparing master mixes
- Nuclease-free pipet tips with aerosol barriers

Equipment

- Permanent marker
- Rotor-Gene Q MDx instrument with 72-well rotor* (QIAGEN, cat. no. 9002035)
- Rotor-Gene Q software version 2.3.5 or later
- Rotor-Gene Q *therascreen* EGFR Assay Package version 3.1.2

  Important: The Rotor-Gene Q *therascreen* EGFR Assay Package version 3.1.2 will only work with Rotor-Gene Q software version 2.3.5 or later.
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction set up (QIAGEN, cat. no. 9018901)
- Dedicated pipets* (adjustable) for sample preparation

* Prior to use, ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

- Dedicated pipets* (adjustable) for PCR master mix preparation
- Dedicated pipets* (adjustable) for dispensing of template DNA
- Benchtop centrifuge* with rotor for 1.5 ml tubes
- Thermomixer, heated orbital incubator, heating block, or water bath capable of incubation at 56°C, 70°C, and 90°C*

* Prior to use, ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

Warnings and Precautions

For in vitro diagnostic use

For prescription use only

For use with Rotor-Gene Q MDx instrument

For use with QIAamp DSP DNA FFPE Tissue Kit Safety information

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

General precautions

- The test is for use with formalin-fixed, paraffin-embedded NSCLC tissue specimens.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Discard sample and assay waste according to your local safety procedures.
- Reagents for the *therascreen* EGFR RGQ PCR Kit are diluted optimally. 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.
- 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.
- Refer to the Rotor-Gene Q MDx instrument user manual for additional warnings, precautions, and procedures.
- Do not use expired or incorrectly stored components.

  Note: Use extreme caution to prevent contamination of the control and reaction mix reagents with the synthetic materials that are contained in the positive control reagent.

  Note: Use individual, dedicated pipets for setting up reaction mixes and adding positive control reagents.

  Note: Perform preparation and dispensing of reaction mixes in an area separate from the one used for the addition of the positive control.

  Note: Do not open the Rotor-Gene Q MDx instrument until the run has finished.

  Note: Do not open Rotor-Gene Q tubes after the run has finished.

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

Reagent Storage and Handling

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

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

Once opened, reagents can be stored in their original packaging at –30 to –15°C for 90 days or until the stated expiration date, whichever comes first. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 8 freeze–thaw cycles.

The reagents must be thawed at ambient temperature for a minimum of 1 hour and a maximum of 4.5 hours. Once the reagents are ready to use, the PCR reactions can be set up. The Rotor-Gene Q tubes, containing the master mixes and the DNA sample, can be loaded onto the Rotor-Gene Q MDx 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: Scorpions (as with all fluorescently labeled molecules) in the reaction mix reagents are light sensitive. Protect control and reaction mix reagents from light to avoid photo bleaching.

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

Attention should be paid to expiration dates and storage conditions printed on the box and labels of all components. Do not use expired or incorrectly stored components.

Specimen Storage and Handling

The *therascreen* EGFR RGQ PCR Kit is for use with DNA samples extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue collected from NSCLC patients. Tumors are heterogeneous in terms of both genotype and phenotype. Mutation-positive tumors can contain wild-type DNA and similarly histology can show regions of non-tumor tissue. All tissue samples should be treated as potentially hazardous.

To prepare tissue samples for DNA purification:

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 purification.
- Scrape the entire tissue area from two sections into labeled microcentrifuge tubes using a fresh scalpel for each sample.

  Note: Use dry scalpels. Do not perform this step in a laminar flow or fume hood.

Label, handle, and store tumor specimens, blocks, slides, samples, and microcentrifuge tubes ready for purification in a controlled fashion according to local procedures.

Store FFPE blocks and slides at room temperature. Slides may be stored at ambient temperature for up to 1 month prior to DNA purification.

Genomic DNA may be stored at 2–8°C for 1 week post purification, or at –25 to –15°C for up to 8 weeks before use.

Procedure

DNA purification

Use the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) with the stipulations described below for purifying genomic DNA from samples prepared from FFPE NSCLC specimens.

Note: The *therascreen* EGFR RGQ PCR Kit has been validated using DNA purified using the QIAamp DSP DNA FFPE Tissue Kit. Do not use any other DNA purification product.

Carry out the DNA purification according to instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook* (Version 1, February 2017) noting the following:

- The QIAamp DSP DNA FFPE Tissue Kit must be used manually only.
- Make sure to use molecular-biology-grade ethanol* for all required steps.
- Use **2 slides** per purification.
- Proteinase K digestions (step 11 in the *QIAamp DSP 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 DSP DNA FFPE Tissue Kit Handbook*) must be performed for **1 hour ± 5 minutes** at 90°C **± 3°C**.
- The samples must be eluted with **120 µl** elution buffer (ATE) from the QIAamp DSP DNA FFPE Tissue Kit.

Genomic DNA may be stored at 2–8°C for 1 week post-purification, or at –25 to –15°C for up to 8 weeks before use.

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

Protocol: DNA sample assessment

This protocol is used to assess the total amplifiable DNA in samples.

Note: DNA sample assessment is not designed to detect the presence of PCR inhibitors as only the total amplifiable DNA in a sample is assessed using the control reaction.

Important points before starting

- To obtain correct results, ensure that the described mixing procedure is performed at each mixing step of the assay setup process.
- Up to 24 samples can be assessed using the control reaction mix available.
- Before beginning the procedure, read the General precautions section.
- Do not vortex the *Taq* DNA polymerase (tube *Taq*) or any mix containing *Taq* DNA polymerase, as this may inactivate the enzyme.
- Pipet *Taq* DNA polymerase by carefully placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.
- Use the control reaction mix (tube CTRL) to assess the DNA prior to testing.

  Note: It is important to use the control reaction mix as described below for this assessment and not spectrophotometry or other alternative methods. Heavily degraded DNA may not amplify even though the primers generate short DNA fragments.
- For efficient use of the reagents in the *therascreen* EGFR RGQ PCR Kit, batch DNA samples as far as possible to create full runs. Testing samples individually or in smaller numbers uses up more reagents and reduces the overall number of samples that can be tested with a single *therascreen* EGFR RGQ PCR Kit.

Things to do before starting

- Ensure that the Rotor-Gene Q *therascreen* EGFR Assay Package software is installed before first use of the Rotor-Gene Q MDx instrument (see "Appendix: Installation of the Rotor-Gene Q *therascreen* EGFR Assay Package", page 82).

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

Procedure

1. Completely thaw the control reaction mix (tube CTRL), nuclease-free water for no template control (tube NTC), and EGFR positive control (tube PC) at ambient temperature (15–25°C) for a minimum of 1 hour and a maximum of 4.5 hours. The times for thawing reagents, PCR setup, and storage before starting the run are indicated in Table 6. When the reagents have thawed, mix them by inverting each tube 10 times to avoid localized concentrations of salts, and then centrifuge briefly to collect the contents at the bottom of the tube.

Table 6. Thawing times, PCR set up times and storage temperatures

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

Note: PCR setup is to be performed at ambient temperature. 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* DNA polymerase (tube *Taq*) to ambient temperature (15–25°C) at the same time as the other reagents (see "Reagent Storage and Handling", page 21). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

2. Prepare sufficient master mix (control reaction mix [tube CTRL] plus *Taq* DNA polymerase [tube *Taq*]) for the DNA samples, one EGFR positive control (tube PC) reaction, and one

nuclease-free water for no template control (tube NTC) reaction) according to the volumes in Table 7. 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 7. Preparation of Control assay master mix

Component	Volume
Control reaction mix (tube CTRL)	19.5 µl x (n+1)*
Taq DNA polymerase (tube *Taq*)	0.5 µl x (n+1)*
Total volume	**20.0 µl/reaction**

* n = number of reactions (samples plus controls). Prepare enough master mix for 1 extra sample (n + 1) to allow for sufficient overage for the PCR setup. The value n should not exceed 24 (plus controls) as 24 is the maximum number of samples that can fit on a run.

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

3. Place the appropriate number of PCR 4-strip tubes (each strip has 4 tubes) in the loading block according to the layout in Table 8. Do not cap the tubes.

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

Table 8. Run layout for DNA sample assessment in the loading block*

Assay									
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	–	–	–	–	–	–

* Each tube should contain a total reaction volume of 25 µl (20 µl of master mix prepared as per Table 7, plus 5 µl of NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position.

4. Cap the tube for the master mix and invert 10 times to mix the master mix followed by brief centrifugation to ensure that the mix is at the bottom of the tube. Immediately add 20 µl of master mix to each PCR strip tube.

   Note: Refer to Table 8 for the tube layout while setting up the master mixes. For DNA sample assessment, control assay master mix should be added to one PC tube, one NTC tube, and one tube for each DNA sample.

5. Immediately add 5 µl nuclease-free water for no template control (tube NTC) to the NTC tube (tube position 2) and cap the tube.
6. Add 5 µl each DNA sample to the sample tubes (tube positions 3–26) and cap the tubes.
7. Add 5 µl EGFR positive control (tube PC) to the PC tube (tube position 1) and cap the tube.
8. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q MDx instrument.
9. **Invert capped tubes 4 times to mix the sample and reaction mix.**
10. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 8) using the marks for orientation.

    Note: If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q MDx instrument is maintained.

11. Place the 72-well rotor into the Rotor-Gene Q MDx instrument. Ensure that the locking ring (supplied with the Rotor-Gene Q MDx instrument) is placed on top of the rotor to secure the tubes during the run.
12. Double-click the ***therascreen*** **EGFR Control Run Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument (Figure 1).

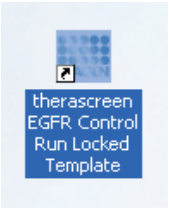

Figure 1. The "therascreen EGFR Control Run Locked Template" icon.

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

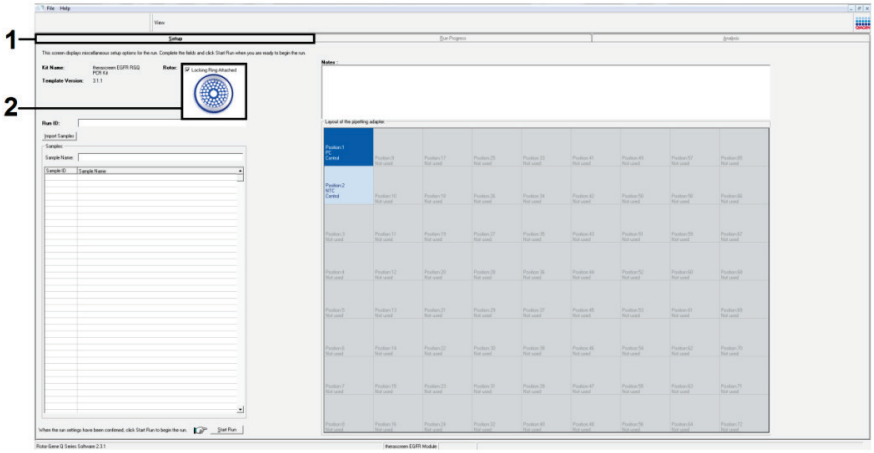

Figure 2. The "Setup" tab and "Locking Ring Attached" box. 1 = "Setup" tab, 2 = "Locking Ring Attached" box.

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

    This will add the sample name to the sample list below and assign the sample a "Sample ID" (1, 2, 3, etc.). In addition, the "Layout of the pipetting adapter" panel on the right side will update to include the sample name (Figure 3). Alternatively, sample names stored in the *.smp (Rotor-Gene Q sample file) or *.csv (comma separated values) format

can be imported using "Import Samples". Sample names will be populated automatically using this method.

Note: In the "Layout of the pipetting adapter" panel, check that addition of the sample name has been 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 adapter" panel, but will appear in full on the report.

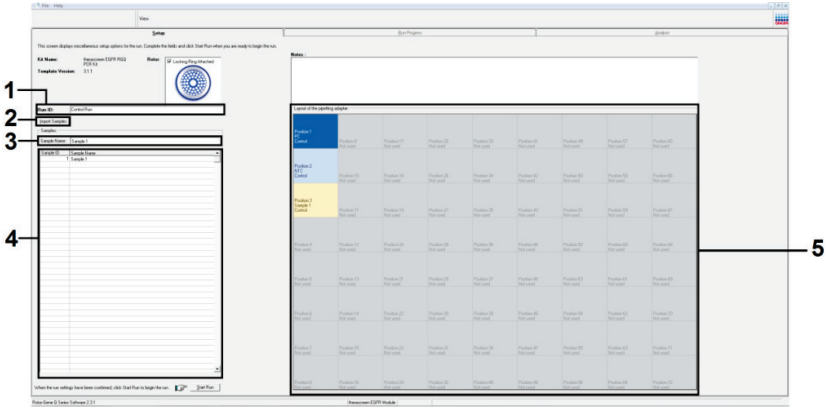

Figure 3. Entering the "Run ID" and "Sample Name". 1 = "Run ID" dialog field, 2 = "Import Sample", 3 = "Sample Name" dialog field, 4 = Sample List, 5 = "Layout of the pipetting adapter" panel.

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

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

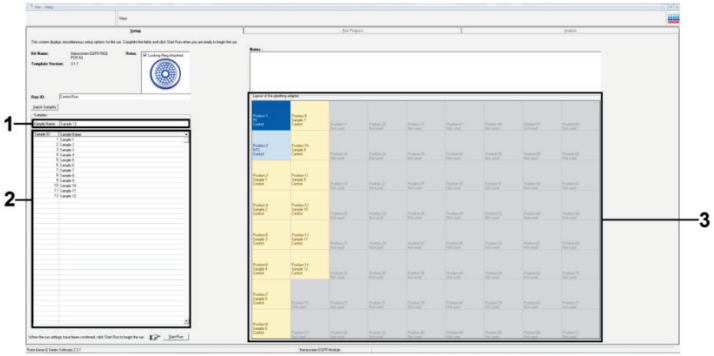

Figure 4. Entering additional sample names in the "Sample Name" dialog field. 1 = "Sample Name" dialog field, 2 = Sample List, 3 = "Layout of the pipetting adapter" panel with additional sample names.

16. When all sample names have been entered, verify that they are correct. Add any additional information in the **Notes** field if necessary and click **Start Run** (Figure 5).

    Note: If any rotor position is unused, a warning will appear (Figure 5 and Figure 6) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube. Check that all unused rotor positions are filled with a capped, empty tube and click **OK** to proceed.

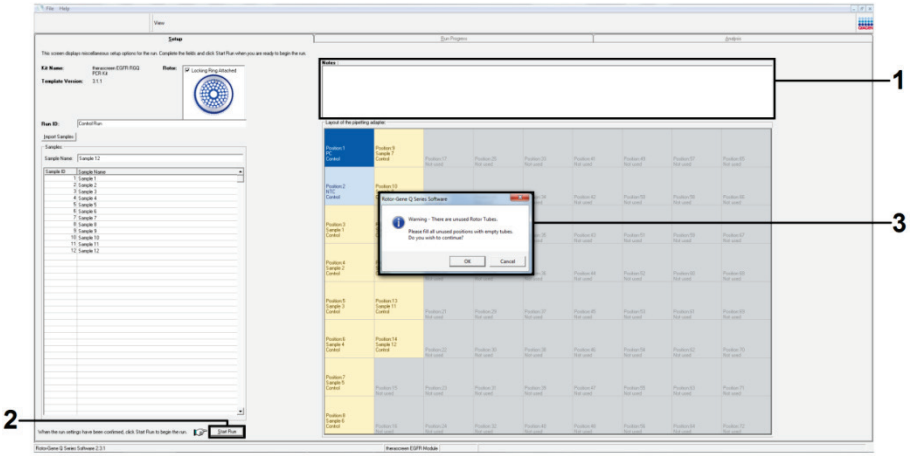

Figure 5. "Notes" dialog field, "Start Run" and "Warning" of unused rotor positions. 1 = "Notes" dialog field, 2 = "Start Run", 3 = Warning of unused rotor positions.

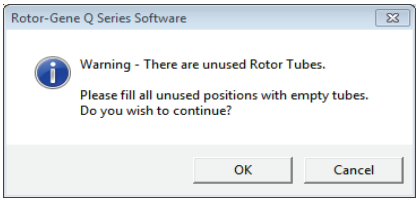

Figure 6. Warning of unused rotor positions.

17. A "Save As" window appears. Select an appropriate file name and save the PCR run as a *.rex run file to the selected location and click **Save** ().

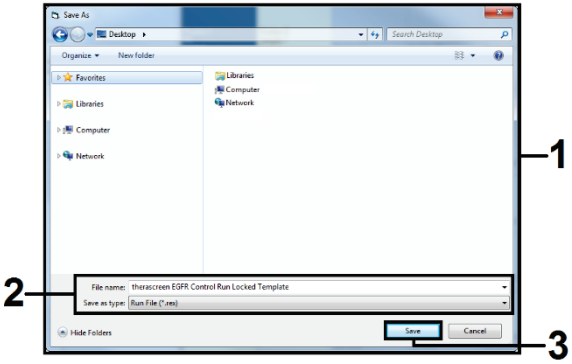

Figure 7. Saving the run file. 1 = "Save As" window, 2 = File name and save as type *.rex file, 3 = "Save".

18. The PCR run starts.

Note: When the run starts, the "Run Progress" tab will open automatically to show the temperature trace and remaining run time (Figure 8).

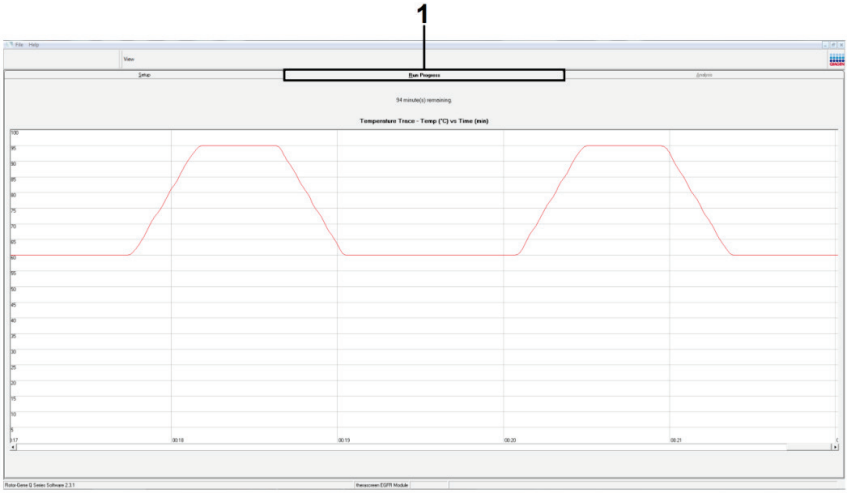

Figure 8 Temperature trace and remaining run time. 1 = The "Run Progress" tab.

19. After the run is finished, the "Analysis" tab will open automatically.

    Note: If the "Analysis" tab fails to open, click the "Analysis" tab (Figure 9).

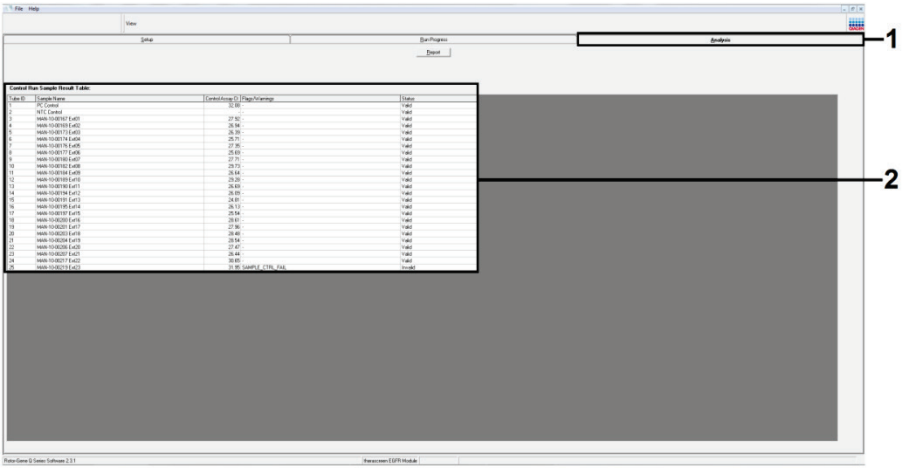

Figure 9. The "Analysis" tab and reporting of results. 1 = "Analysis" tab, 2 = "Control Run Sample Result Table".

Control results will be reported as follows in the "Control Run Sample Result Table" (Figure 9).

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

- **Run controls (PC and NTC, tube positions 1 and 2 respectively)**. If the results are within acceptable ranges, each will display **Valid** otherwise an **Invalid** result will appear.
- **Sample control reaction C_T>31.10, will display "Invalid"**. Quantity of DNA is not sufficient for mutation analysis. Retest the sample. If the quantity of DNA is still insufficient, extract two further FFPE tissue sections, if available (see "Troubleshooting Guide", page 75).
- **Sample control reaction C_T<23.70, will display "Invalid"**. DNA concentration is too high for mutation analysis. Dilute with Nuclease-Free Water for Dilution (tube Dil.) and retest. Dilute to a C_T of 23.70–31.10. A 1:1 dilution 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) will display "Valid"**. DNA concentration is suitable for mutation analysis.

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

Report files can be produced by clicking **Report**. The "Report Browser" window will appear. Select **EGFR Analysis Report** under "Templates", then click **Show** (Figure 10).

Note: Reports can be saved to an alternative location in Web Archives format by clicking **Save As** on the top left corner of each report.

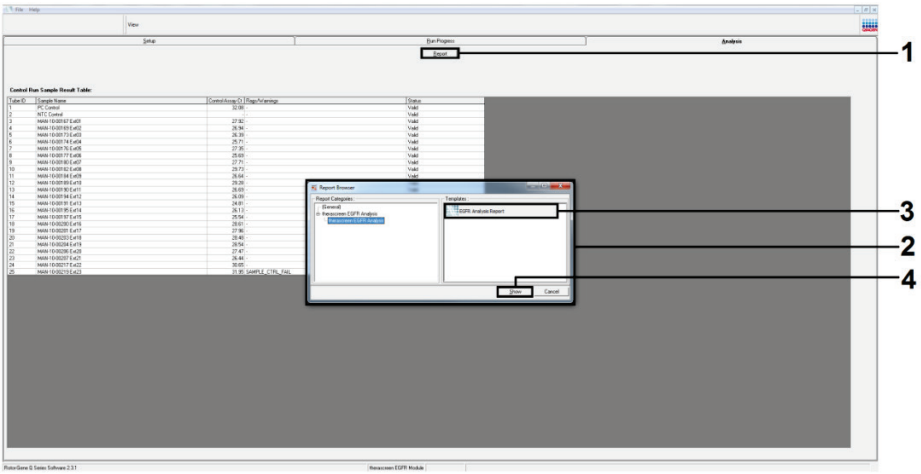

Figure 10. Selecting the "EGFR Analysis Report". 1 = "Report", 2 = "Report Browser" window, 3 = "EGFR Analysis Report" selection, 4 = "Show".

Protocol: Detection of EGFR mutations

This protocol is for the detection of EGFR mutations.

Important points before starting

- To obtain correct results, ensure that the described mixing procedure is performed at each mixing step of the assay setup process.
- A sample can be tested using the EGFR mutation assays once it has passed the sample assessment.
- For efficient use of the *therascreen* EGFR RGQ PCR Kit, samples must be grouped into batches of 7 (to fill the 72-well rotor). Smaller batch sizes will mean that fewer samples can be tested with the *therascreen* EGFR RGQ PCR Kit.
- The sample must be tested using all reaction mixes provided in the *therascreen* EGFR RGQ PCR Kit.
- Do not vortex the *Taq* DNA polymerase (tube *Taq*) or any mix containing *Taq* DNA polymerase, as this may inactivate the enzyme.
- Pipet *Taq* DNA polymerase 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 Rotor-Gene Q *therascreen* EGFR Assay Package software is installed before first use of the Rotor-Gene Q MDx instrument (see "Appendix: Installation of the Rotor-Gene Q *therascreen* EGFR Assay Package", page 82).
- Before each use, all reagents need to 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 contents at the bottom of the tube.

- Ensure that *Taq* DNA polymerase (tube *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. Completely thaw all reaction mix tubes, nuclease-free water for no template control (tube NTC), and EGFR positive control (tube PC) at ambient temperature (15–25°C) for a minimum of 1 hour. The times for thawing reagents, PCR setup, and storage before starting the run are indicated in Table 9. When the reagents have thawed, **mix them by inverting each tube 10 times** to avoid localized concentrations of salts and then centrifuge briefly to collect the contents at the bottom of the tube.

Table 9. Thawing times, PCR set up times, and storage temperatures

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

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

Note: Bring the *Taq* DNA polymerase (tube *Taq*) to ambient temperature (15–25°C) at the same time as the other reagents (see "Reagent Storage and Handling", page 21). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

2. Label 8 microcentrifuge tubes (not provided) according to each corresponding reaction mix shown in Table 10. Prepare sufficient master mixes (control or mutation reaction mix [tube CTRL, T790M, Deletions, L858R, L861Q, G719X, S768I, or Insertions] plus *Taq* DNA polymerase [*Taq*]) for the DNA samples, one EGFR positive control (tube PC) reaction, and one nuclease-free water for no template control (tube NTC) reaction according to the volumes in Table 10. Include reagents for one extra sample to allow sufficient overage for the PCR setup.

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

Table 10. Preparation of assay master mixes*

Assay	Reaction mix tube	Volume of reaction mix	Volume of *Taq* DNA polymerase (tube *Taq*)
Control	CTRL	19.50 µl x (n+1)	0.50 µl x (n+1)
T790M	T790M	19.50 µl x (n+1)	0.50 µl x (n+1)
Deletions	Del	19.50 µl x (n+1)	0.50 µl x (n+1)
L858R	L858R	19.50 µl x (n+1)	0.50 µl x (n+1)
L861Q	L861Q	19.50 µl x (n+1)	0.50 µl x (n+1)
G719X	G719X	19.50 µl x (n+1)	0.50 µl x (n+1)
S768I	S768I	19.50 µl x (n+1)	0.50 µl x (n+1)
Insertions	Ins	19.50 µl x (n+1)	0.50 µl x (n+1)

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

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

3. Place the appropriate number of PCR 4-strip tubes (each strip has 4 tubes) in the loading block according to the layout in Table 11. Do not cap the tubes.

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

4. Cap the tube for the master mix and **invert 10 times** to mix the master mix followed by brief centrifugation to ensure the mix is at the bottom of the tube. Immediately add 20 µl master mix to each appropriate PCR strip tube.

   Note: Refer to Table 11 for the tube layout while setting up the reaction mixes. For detection of EGFR mutations, master mixes should be added to 8 PC tubes, 8 NTC tubes, and 8 tubes for each DNA sample.

Table 11. Run layout for the EGFR Assay*

	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

* Each tube should contain a total reaction volume of 25 µl (20 µl master mix prepared as per Table 10, plus 5 µl NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position.

5. Immediately add 5 µl of nuclease-free water for no template control (tube NTC) to the NTC tubes (tube positions 9–16) and cap the tubes.
6. Add 5 µl each DNA sample to the sample tubes (tube positions 17–72) and cap the tubes.
7. Add 5 µl EGFR positive control (tube PC) to the PC tubes (tube positions 1–8) and cap the tubes.
8. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5 and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q MDx instrument.
9. **Invert capped tubes 4 times to mix the sample and reaction mix.**
10. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 11) using the marks for orientation.

    Note: A maximum of 72 samples can be included in each PCR run. If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q MDx instrument is maintained.

11. Place the 72-well rotor into the Rotor-Gene Q MDx instrument. Ensure that the locking ring (supplied with the Rotor-Gene Q MDx instrument) is placed on top of the rotor to secure the tubes during the run.
12. Open the Rotor-Gene Q software according to step 12.
13. Double-click the **therascreen EGFR Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument (Figure 11).

Figure 11. The "therascreen EGFR Locked Template" icon.

14. The "Setup" tab appears as the default (Figure 12). Ensure that the locking ring is properly attached and check the **Locking Ring Attached** box. Close the lid of the Rotor-Gene Q MDx instrument.

Figure 12. The "Setup" tab and "Locking Ring Attached" box. 1 = "Setup" tab, 2 = Checked "Locking Ring Attached" box.

15. Enter the run ID in the **Run ID** field according to your local naming convention. Enter the sample name in the **Sample Name** field according to your local naming convention and press the **Return** key. This will add the sample name to the sample list below and assign the sample a "Sample ID" (1, 2, 3, etc.). In addition, the "Layout of the pipetting adapter" panel on the right side will update to include the sample name (Figure 13).

    Note: In the "Layout of the pipetting adapter" panel, check that addition of the sample name has been highlighted by a change in color and that all eight assays in the column under the sample circle are highlighted (Figure 13).

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

    Note: Sample names with more than 8 characters may not be completely displayed in the "Layout of the pipetting adapter" panel, but will appear in full on the report.

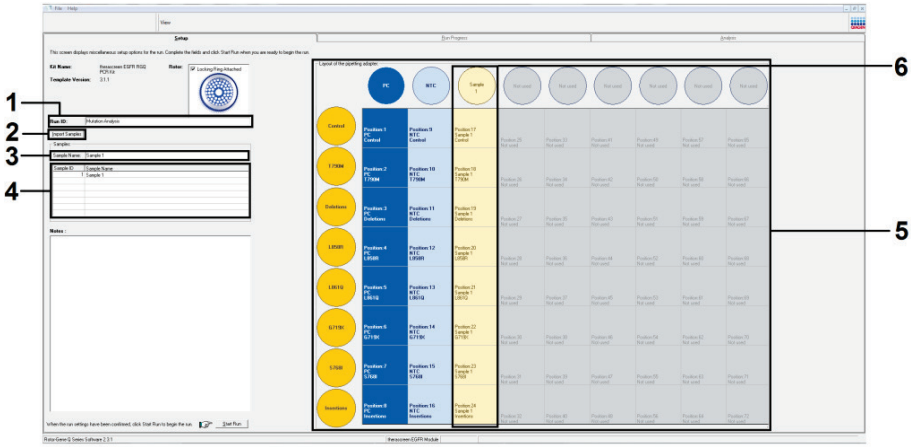

Figure 13. Entering the "Run ID" and "Sample Name". 1 = "Run ID" dialog field, 2 = "Sample Import", 3 = "Sample Name" dialog field, 4 = Sample List, 5 = "Layout of the pipetting adapter" panel, 6 = Highlighted sample circle and column of eight assays underneath.

16. Repeat step 14 to enter the names of all additional samples (Figure 14).

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

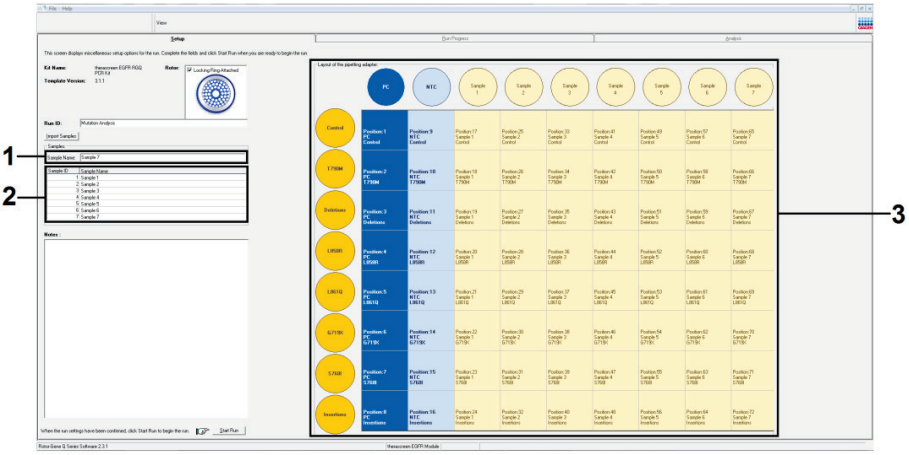

Figure 14. Entering additional sample names in the "Sample Name" dialog field. 1 = "Sample Name" dialog field, 2 = Sample List, 3 = "Layout of the pipetting adapter" panel with additional sample names.

17. When all sample names have been entered, verify that they are correct. Add any additional information in the **Notes** field if necessary, then click **Start Run** (Figure 15).

    Note: If any rotor position is unused, a "Warning" will appear (Figure 15 and Figure 16) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube. Check that all unused rotor positions are filled with a capped, empty tube and click **OK** to proceed.

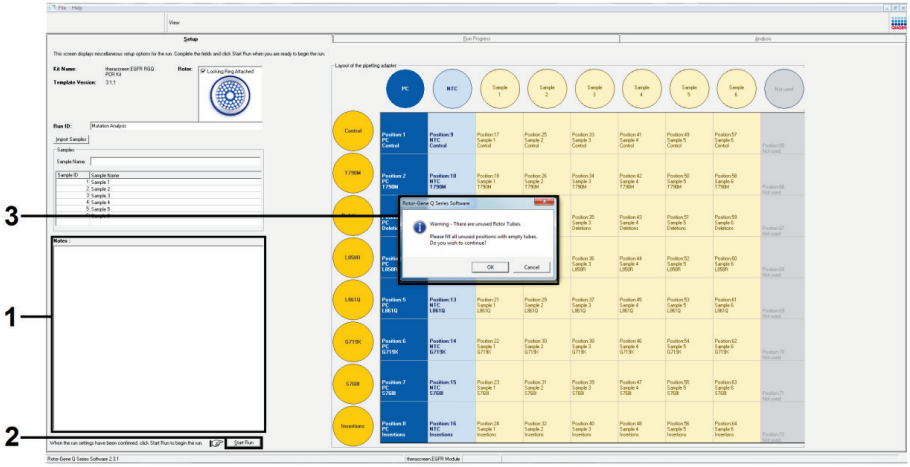

Figure 15. "Notes" dialog field, "Start Run" and "Warning" of unused rotor positions. 1 = "Notes" dialog field, 2 = "Start Run", 3 = Warning of unused rotor positions.

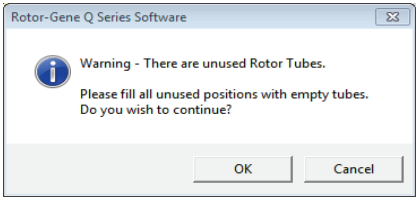

Figure 16. Warning of unused rotor positions.

18. A "Save As" window appears. Select an appropriate file name and save the PCR run as a *.rex run file to the selected location (Figure 17).

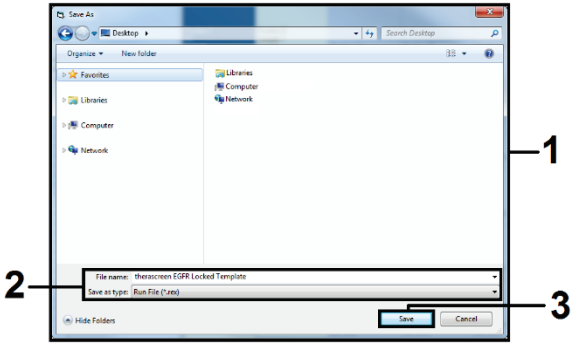

Figure 17. Saving the run file. 1 = "Save As" window, 2 = File name and save as type *.rex file, 3 = "Save".

19. The PCR run starts.

    Note: When the run starts, the "Run Progress" tab will open automatically to show the temperature trace and remaining run time (Figure 18).

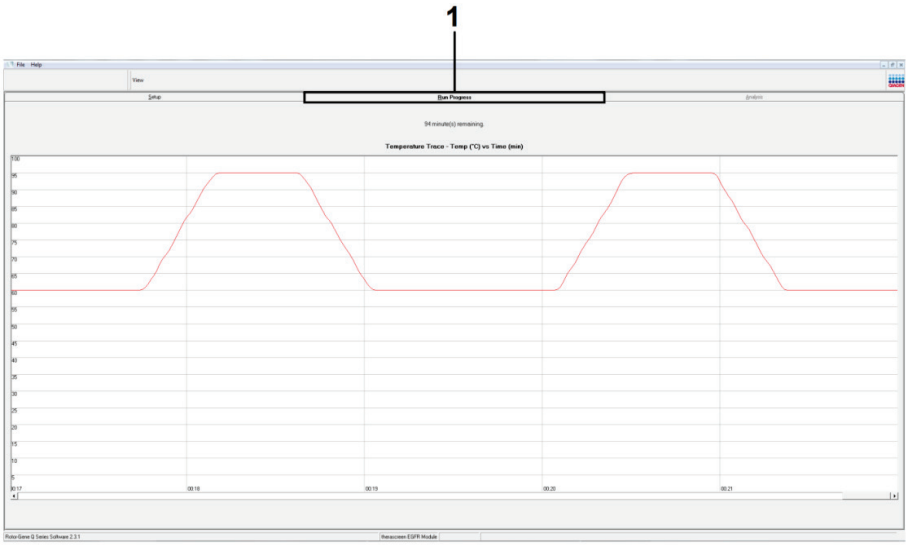

Figure 18. Run. 1 = the "Run Progress" tab.

20. After the run is finished, the "Analysis" tab will open automatically.

Note: If the "Analysis" tab fails to open, click the "Analysis" tab (Figure 19).

Note: An explanation of the calculation method is presented in the "Results" section, page 47.

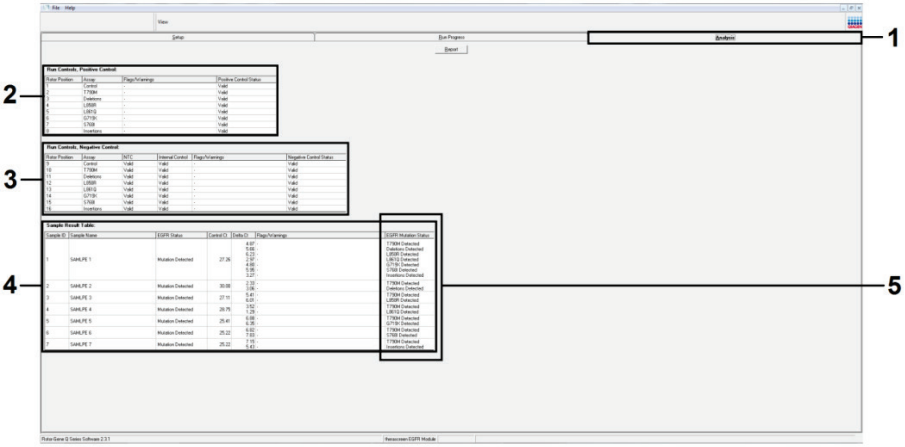

Figure 19. The "Analysis" tab and reporting of results. 1 = "Analysis" tab, 2 = "Run Controls, Positive Control" panel, 3 = "Run Controls, Negative Control" panel, 4 = "Sample Result Table", 5 = "EGFR Mutation Status" column.

Assay results will be reported as follows (Figure 19).

The "Run Controls, Positive Control" panel. If the results are within acceptable range, the "Positive Control Status" will display **Valid**, otherwise an **Invalid** result will appear.

The "Run Controls, Negative Control" panel. 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.

The "Sample Result Table" panel. Specific mutations will be reported for the Mutation Positive samples under the "EGFR Mutation Status" column.

Report files can be produced by clicking **Report**. The "Report Browser" window will appear. Select **EGFR Analysis Report** under "Templates", then click **Show** (Figure 20).

Note: Reports can be saved to an alternative location in Web Archives format by clicking **Save As** on the top left corner of each report.

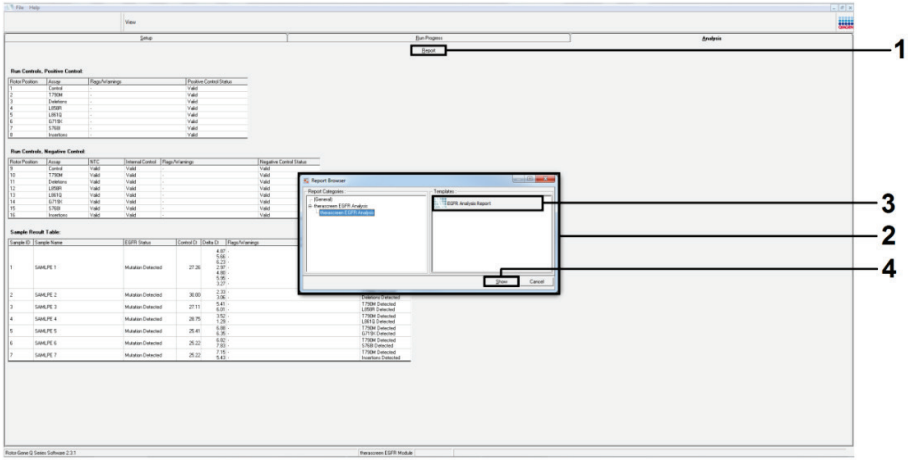

Figure 20. Selecting the "EGFR Analysis Report". 1 = "Report", 2 = "Report Browser" window, 3 = "EGFR Analysis Report" selection, 4 = "Show".

Results

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

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

The same run parameters are used for both the DNA sample assessment with the control reaction mix and for detection of EGFR mutations using the mutation reaction mixes.

1. Hold at 95°C for 15 minutes to activate the *Taq* DNA polymerase.
2. PCR for 40 cycles of 95°C for 30 seconds to denature and 60°C for 1 minute to anneal and extend.

By using the control reaction to assess the DNA sample, it is possible to determine if the samples contain DNA levels that are suitable for analysis and which samples require dilution prior to mutation analysis based on the C_T values obtained.

Based on predetermined analytical C_T and ΔC_T values, the Rotor-Gene Q software qualitatively determines the mutation status of the DNA samples and reports which samples contain which mutation.

The analysis and mutation calls are performed automatically by the Rotor-Gene Q *therascreen* EGFR Assay Package once a run is completed. The following information explains how the Rotor-Gene Q *therascreen* EGFR Assay Package makes the analysis and mutation calls.

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 amplification.

The Rotor-Gene Q software interpolates fluorescence signals between any two recorded values. The C_T values can therefore be any 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 channel and 0.02 for the yellow channel. These values are configured in the Rotor-Gene Q *therascreen* EGFR Assay Package for both the green and yellow channels. The threshold values were defined during development of the *therascreen* EGFR RGQ PCR Kit.

The run controls (positive control and NTC, and the internal control within each reaction mix) are assessed to ensure that acceptable C_T values are met and the reactions are performing correctly.

Sample ΔC_T values are calculated as the difference between the mutation assay C_T and control assay C_T from the same sample. Samples are classed as mutation positive if they give a ΔC_T within the ΔC_T cutoff range for that assay (Table 12). Above the ΔC_T range, the sample would be reported as "No Mutation Detected" as it 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. Below the ΔC_T range, the sample would be reported as "Invalid".

Table 12. Mutation assay ΔC_T cutoff range

Mutation assay	ΔC_T cutoff range
T790M	–10.00 ≥ to ≤7.40
Del*	–10.00 ≥ to ≤8.00
L858R	–10.00 ≥ to ≤8.90
L861Q	–10.00 ≥ to ≤8.90
G719X	–10.00 ≥ to ≤8.90
S768I	–10.00 ≥ to ≤8.90
Ins†	–10.00 ≥ to ≤8.00

* Exon 19 deletions.
† Exon 20 insertions.

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

The assay results will be 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. Other possible results that may be displayed are discussed in the "Protocol: DNA sample assessment" (page 25), and "Troubleshooting Guide" (page 75) sections of this Instructions for Use.

A tumor may contain more than one mutation. In such instances, more than one mutation will be reported.

Quality Control

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

Limitations of the Procedure

Samples with results reported as "No Mutation Detected" may harbor EGFR mutations not detected by the *therascreen* EGFR RGQ PCR Kit.

Detection of mutations is dependent on sample integrity and the amount of amplifiable DNA present in the specimen. The procedure should be repeated in the event that the initial assessment of the DNA in the sample indicates that the quantity is either not sufficient or too high for mutation analysis.

The *therascreen* EGFR RGQ PCR Kit is used in a polymerase chain reaction (PCR) procedure. As with all PCR procedures, samples may be contaminated by external sources of DNA in the test environment and the DNA in the positive control. Use caution to avoid contamination of samples and reaction mix reagents.

The *therascreen* EGFR RGQ PCR Kit is only validated for use with formalin-fixed, paraffin-embedded NSCLC tissue.

The *therascreen* EGFR RGQ PCR Kit is only validated for use with the QIAamp DSP DNA FFPE Tissue Kit.

The *therascreen* EGFR RGQ PCR Kit is only validated for use when all reaction mixes are used.

Only the Rotor-Gene Q MDx instrument has been validated for use with the *therascreen* EGFR RGQ PCR Kit. No other thermal cycler with real-time optical detection can be used with this product.

Though infrequent, mutations within the genomic DNA regions of the EGFR gene covered by the primers or probes used in the *therascreen* EGFR RGQ PCR Kit may result in failure to detect presence of a mutation in exons 18, 19, 20, and 21 of the EGFR oncogene (results of "No Mutation Detected").

The primers in the EGFR Deletions Reaction mix have been designed to target multiple Exon 19 deletions, spanning nucleotides *55174772* to *55174795* (GRCh38 chr*7*), a range of 23 bp.

While the Exon 19 deletions assay has been analytically validated and demonstrated to detect 14 specified deletions within Exon 19 (see list in both Table 1 and Table 3 of this handbook), it is, however, possible for additional mutations (including, but not limited to, additional Exon 19 deletions, Exon 19 insertions, and the L747P mutation*) to be amplified by the Deletions primer set.

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

The *therascreen* EGFR RGQ PCR Kit is a qualitative test. The test is not for quantitative measurements of percent mutation.

Performance of the *therascreen* EGFR RGQ PCR Kit is unknown if microbial contamination is introduced during assay procedures.

* Mutations determined to cross-react with the *therascreen* EGFR RGQ PCR Kit are detailed in Table 15, page 56, of the "Performance Characteristics" section.

Performance Characteristics

Analytical Performance

The specific performance characteristics of the QIAGEN *therascreen* EGFR RGQ PCR Kit were determined by studies using formalin-fixed, paraffin-embedded (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. Bi-directional Sanger sequencing and massively parallel sequencing were used to select the specimens for the following studies and determine the percentage of mutation in NSCLC FFPE samples. The similarity between FFPE clinical specimens and FFPE cell lines was demonstrated by comparing assay amplification efficiencies (AE) between the two sample types and by assessing the limit of detection (LoD) for the specific mutation assays. FFPE cell lines were sectioned and processed similar to FFPE clinical specimens. DNA was extracted and tested according the instructions for use.

Analytical Sensitivity – Limit of Blank (LoB)

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 FFPE EGFR wild-type cell line DNA were evaluated. The results demonstrated no positive mutation calls with no-template control (NTC) samples and FFPE wild-type samples.

Analytical Sensitivity – Limit of Detection (LoD)

The LoD is the minimum percentage of mutant DNA that can be detected in a background of wild-type DNA when the total amplifiable DNA (within the input range) produced correct mutation calls at 95% for each mutation positive sample (C95).

The DNA input working range for the assay is defined by the control C_T at pre-specified range of 23.70 to 31.10 (also refer to "Analytical Sensitivity – Control C_T Range", page 55).

The LoD was determined at low DNA input (control C_T approximately 30.10) levels.

The NSCLC FFPE clinical specimens or FFPE cell lines were used in the LoD study. The mutant DNA, extracted from the FFPE clinical specimens or FFPE cell lines, was diluted in a background of wild-type DNA in order to create a series of samples containing different percentages of mutant DNA while keeping the total DNA constant either at the high or low DNA input level. At each dilution level (% mutation), 24 replicates were evaluated using multiple *therascreen* EGFR RGQ PCR Kit lots.

Logistic regression analysis was applied to each mutation individually using each of the low and high input DNA datasets. The LoD was determined for each EGFR mutation at either the low or high DNA input levels. The final LoD claims listed in the tables below indicate the percentage of mutation that gave a predicted probability of correct calls of 95% for each of the 20 (see note [**] under Table 1) mutations.

The final LoD claims for EGFR mutations were fully supported by results from the reproducibility study conducted at low DNA input (refer to "Repeatability and reproducibility", page 62). Among the 20 (see note [**] under Table 1) EGFR mutations detected by the *therascreen* EGFR RGQ PCR Kit, safety and efficacy of GILOTRIF has been established for the 17 (see note [**] under Table 1) mutations listed in Table 13, but has not been established for the 3 mutations listed in Table 14. Refer to GILOTRIF drug labeling for more details.

Table 13. Sensitivity of the *therascreen* EGFR RGQ PCR Kit – safety and efficacy of GILOTRIF established

Exon	Mutation	Cosmic ID	Base change	Sample type*	Final LOD claim (% mutation) Low DNA input
18	G719A	6239	2156G>C	CL	32.5‡
19	Deletions	6220	2238_2255del18	CL	2.70
		6223†	2235_2249del15	CL+CS	6.40
		6225†	2236_2250del15	CL+CS	6.50‡
		6254**	2239_2253del15	CS	10.20‡
		6255	2239_2256del18	CS	0.81‡
		12369**	2240_2254del15	CS	4.94
		12370	2240_2257del18	CS	8.10
		12382	2239_2248TTAAGAGAAG>C	CS	1.45‡
		12383	2239_2251>C	CS	4.58
		12384	2237_2255>T	CS	7.54‡
		12387	2239_2258>CA	CL	4.91
		12419	2238_2252>GCA	CL	16.87
		12422	2238_2248>GC	CL	3.24
		13551	2235_2252>AAT	CL	4.24
20	S768I	6241	2303G>T	CL	7.66
21	L858R	6224†	2573T>G	CL+CS	5.94
21	L861Q	6213	2582T>A	CL	9.24‡

* CS denotes FFPE clinical specimen; CL denotes FFPE cell line.

† LoD was determined using both FFPE clinical specimens and FFPE cell line at low DNA input level for these four EGFR mutations in Table 13 and Table 14, which cover 68.71% of the reported EGFR mutations.

‡ Final LoD % mutation claims are based on results from reproducibility study.

** See note (**) under Table 1.

Analytical Sensitivity – Control C_T Range

A set of 417 sectioned FFPE clinical specimen blocks were assayed and characterized using the *therascreen* EGFR RGQ PCR Kit. Among these, 400 samples were wild-type and 17 samples were mutation-positive as determined by bi-directional Sanger sequencing. EGFR mutations representing 5 out of the 7 mutation assays in the *therascreen* EGFR RGQ PCR Kit were covered in this study. Samples for the remaining 2 mutation assays (i.e., G719X and S768I) were not available. Control assay C_T value data was not normally distributed; Therefore, nonparametric methods were used. The nonparametric one-sided tolerance intervals used for each boundary were selected at 90% coverage with 99% confidence. The boundaries selected were further rounded following considerations in accordance with the user needs and risk management. The final control reaction C_T working range selected for the EGFR Kit is determined to be 23.70 to 31.10 C_T.

Analytical Sensitivity – Upper ΔC_T Cutoff Values

A risk-based approach was taken with regard to false positive rates when setting the assay cutoff values, and estimated LoB values were used as one component in developing cutoff values. Refer to "Results" section, page 47, for the upper cutoff values selected.

Table 14. Sensitivity of the *therascreen* EGFR RGQ PCR Kit – safety and efficacy of GILOTRIF not established

Exon	Mutation	Cosmic ID	Base change	Sample type*	Final LoD claim (% mutation)	
					Low DNA input	High DNA input
20	T790M	6240†	2369C>T	CS	8.16	6.32
20	Insertion	12377	2319_2320insCAC	CL	3.72‡	N/A
20	Insertion	12378	2310_2311insGGT	CL	19.96‡	N/A

* CS denotes FFPE clinical specimen; CL denotes FFPE cell line.

† LoD was determined using both FFPE clinical specimens and FFPE cell line at low DNA input level for these four EGFR mutations in Table 13 and Table 14, which cover 68.71% of the reported EGFR mutations.

‡ Final LoD % mutation claims are based on results from reproducibility study.

N/A: Not available.

Analytical Sensitivity – Effect of DNA Input on ΔC_T

The DNA input level is defined as the total quantity of amplifiable EGFR DNA in a sample and is determined by the C_T values from the control reaction. To demonstrate that the performance of the *therascreen* EGFR RGQ PCR Kit is consistent over the total DNA input (control C_T) range of the assay, mutations detected by the *therascreen* EGFR RGQ PCR Kit were tested, including all seven EGFR mutation assays. DNA extracted from FFPE cell lines was used to prepare pools of DNA at the lower end of the control reaction working range. The target C_T values were set for dilution 1 (100% or undiluted) for each mutation of C_T at approximately 24.70. This pool of DNA was used to generate six equally spaced dilution levels across and beyond the working range, resulting in a dilution of approximately 1 in 3 (i.e., each subsequent dilution level contained approximately threefold less DNA). The final dilution point was outside of the lowest DNA input level of the working range, C_T approximately 32–33 C_T. 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 and passed the pre-specified acceptance criteria for the study.

Linearity – Amplification Efficiency as a Function of DNA Input

The linearity and amplification efficiency of PCR for each mutation assay, relative to the control reaction, across the working range of the *therascreen* EGFR RGQ PCR Kit, was investigated. Amplification efficiency was calculated using linear regression with assay C_T as the response variable and $\log_2$ relative DNA input level as the explanatory variable for EGFR mutations detected by the *therascreen* EGFR RGQ PCR Kit including all the 7 EGFR mutation assays and the control reaction. EGFR mutations were tested targeting the lower end of the control reaction working range (approximately 25 C_T, high DNA input) and were serially diluted with ATE buffer, effectively diluting the input DNA and mutant DNA equally. The final dilution point was outside of the lowest DNA input level of the working range (C_T approximately 32–33C_T). The amplification efficiency of the control assay compared to the mutation assay indicates that the ΔC_T, and thus mutation call, is consistent across the working range of the assay.

Linearity – Amplification Efficiency as a Function of % Mutation

The objective of this study was to evaluate the linearity of each mutant assay across the working range of the assay when the total amount of DNA is held constant but the percentage of mutant DNA is varied. To maintain an equivalent control C_T across the dilution series, EGFR-mutation-positive FFPE cell line DNA was diluted with the wild-type FFPE cell line DNA. Dilution series at both the high DNA input (control C_T approximately 26) and low DNA input (control C_T approximately 29–30) were tested. For each EGFR mutation, pools of DNA sufficient for six replicates at each dilution level were prepared. The C_T and ΔC_T data for each mutation at each dilution level were calculated. The control C_T values corresponding to either approximately 26 C_T or approximately 29–30 C_T were consistent over the dilution series of each mutation. A linear regression model was fitted to estimate the difference in mean ΔC_T between the two DNA input levels. A plot of the ΔC_T values was generated showing the data for both high and low DNA input levels on the same plot. The slope and 95% confidence intervals (95% CI) were reported. The study results showed that dilution of mutations in a background of a constant amount of total DNA resulted in amplification efficiencies that are mostly comparable (± 10%) to the mutation amplification efficiency determined in the above

linearity study (Linearity — amplification efficiency as a function of DNA input). Amplification efficiencies differing close to or greater than 10% are noted for Del6220, Del6223, G719A, Ins12377, and L861Q mutations.

Analytical Specificity – Primer and Probe Specificity

The primers and probes have been designed to avoid any known EGFR polymorphisms. A specificity analysis was conducted using the Basic Local Alignment Search Tool (BLAST) to ensure that the primers used in the *therascreen* EGFR RGQ PCR Kit would amplify only human EGFR sequences and not sequences from other species or non-EGFR human sequences (e.g., pseudogenes). No nonspecific amplification is predicted from non-EGFR genes. In addition, alignments of pairs of oligonucleotides (primers, probes, and templates) used in the *therascreen* EGFR RGQ PCR Kit were performed to ensure there is no unexpected binding that could lead to nonspecific amplification. There was no significant homology between the various reagents.

Analytical Specificity – Cross-Reactivity to other EGFR Mutations

Cross-reactivity of the *therascreen* EGFR RGQ PCR Kit to other EGFR mutations was observed in the Phase 3 clinical trial specimens, FFPE cell lines, EGFR plasmids, and investigations in long oligonucleotides. The *therascreen* EGFR RGQ PCR Kit gave "Mutation Detected" results for the following EGFR mutations in the specific sample types indicated in Table 15. Analytical performance of the *therascreen* EGFR RGQ PCR Kit in detecting these mutations has not been evaluated for its intended use.

Table 15. Mutations determined to cross-react with the *therascreen* EGFR RGQ PCR Kit

Mutation	COSMIC ID*	Sample type
Exon 19 – mutation assay Del		
2237_2251del15	12678	FFPE clinical trial specimen†
2239_2247del9	6218	FFPE cell line
2236_2253del18	12728	FFPE cell line
2237_2254del18	12367	FFPE cell line
2240_2251del12	6210	FFPE cell line
L747P‡	24267	In silico
Exon 18 – mutation assay G719X		
G719S	6252	FFPE clinical trial specimen§
G719C	6253	Plasmid
Exon 20 – mutation assay Ins		
Insertion	12376	FFPE cell line
Exon 21 - mutation assay L858R		
L858Q¶	29578	Long oligonucleotides

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

† The exon 19 deletion 12678 was observed in non-randomized study population of the 1200.32 clinical trial.

‡ The exon 19 L747P mutation was observed to cross-react with the Exon 19 Deletion reaction mix (12). L747P is a rare acquired mutation that may confer resistance to TKI treatment (13).

§ The exon 18 G719S mutation was observed in randomized study population of the 1200.32 clinical trial.

¶ The rare mutation L858Q was observed to cross-react with the L858R reaction mix by in-silico analysis and analysis of long oligonucleotides.

Analytical Specificity – Cross-Reactivity/Exclusivity

Nonspecific amplification/cross-reactivity: Wild-type EGFR DNA

To address the amount of nonspecific amplification of wild-type EGFR DNA by reaction mixes designed to amplify specific mutations, 60 replicates of wild-type FFPE cell line DNA at approximately the highest concentration of amplifiable DNA input level (control C_T approximately 25) were evaluated using the *therascreen* EGFR RGQ PCR Kit. These results demonstrated that the lowest ΔC_T values exceeded the established cutoff values indicating that nonspecific amplification was not observed.

Nonspecific amplification/exclusivity: Mutation-positive EGFR DNA

The exclusivity of the *therascreen* EGFR RGQ PCR Kit is intended to discriminate between mutation negative and mutation positive status. Mutant samples with a high concentration of input DNA (control C_T approximately 25) were tested against all reaction mixes by preparing DNA samples from FFPE cell lines. Sixty (60) replicates of each mutation sample were evaluated. The results demonstrated that there is no impact due to the cross-reactivity between mutant reactions as the minimum ΔC_T values were all higher than the respective assay cutoff values for all non-matching reaction mixes and mutant DNA samples.

Interference – Effects of necrotic tissue

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

Interference – Exogenous substances

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

Lot-to-lot reproducibility

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

Specimen handling – Reproducibility

To assess sample handling variability as part of the *therascreen* EGFR RGQ PCR Kit test system process, the reproducibility of the QIAamp DSP DNA FFPE Tissue Kit was examined using sections taken from three FFPE specimen blocks: one containing an exon 19 deletion mutation (2235-2249 del15), one containing the exon 21 L858R mutation (2573T>G), and one that is wild-type. For each specimen, purifications were carried out in duplicate at each test site and tested on 3 nonconsecutive days over a period of 6 days across 3 sites, yielding a total of 18 data points per specimen. At each site, two 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. One Rotor-Gene Q MDx instrument was used to conduct the testing at Site 1 and 2 Rotor-Gene Q MDx instruments were used at Sites 2 and 3. 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.

Repeatability and reproducibility

The repeatability 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. Reproducibility was conducted across 3 sites (i.e., United Kingdom, Germany, and USA). At each site, samples were tested in duplicate (for within-run repeatability assessment), on 2 different Rotor-Gene Q MDx instruments, using 2 operators and 2 *therascreen* EGFR RGQ PCR Kit lots (3 lots across 3 sites) over a total of 16 days. Reproducibility for each individual mutation was conducted over nonconsecutive days at each site.

Two sets of samples were prepared at different mutations levels, both sets having low DNA input levels where a control C_T value of approximately 30.10 was targeted. There were no "Mutation Detected" results in 84 valid tests of wild-type sample, producing 100% correct calls. The percentage of correct calls ranged from 96–100% for mutant samples tested at 1–3x LoD across sites.

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% in all EGFR mutations tested. Across all mutant panel members, the %CV was ≤8.33% for between-lots, between lots, between days, and between runs. The %CV for within-run (repeatability) ranged from 5.99% to 13.49%.

Table 16. Assay reproducibility – proportion of correct calls for EGFR mutation tested

Exon	Mutation	COSMIC ID	% Mutation tested	% Mutation tested relative to final LoD claim	Number of valid results, N	Correct calls, N	% Correct calls	% Correct call lower one-sided 95% CI
19	Deletions	6220*	5.69%	2–3x LoD	96	96	100	96.93
19	Deletions	6223	15.99%	2–3x LoD	95	95	100	96.90
19	Deletions	6225	7.06%	1–2x LoD	95	91	95.79	90.62
19	Deletions	6254**	10.02%	LoD	92	92	100	96.80
19	Deletions	6255	0.81%	LoD	96	94	97.92	93.59
19	Deletions	12369**	9.29%	1–2x LoD	95	95	100	96.90
19	Deletions	12370	8.06%	LoD	63†	62	98.41	92.69
19	Deletions	12382	1.45%	LoD	95	92	96.84	92.04
19	Deletions	12383	8.43%	1–2x LoD	93	93	100	96.83
19	Deletions	12384	7.54%	LoD	92	92	100	96.80
19	Deletions	12387*	9.53%	1–2x LoD	95	95	100	96.90
19	Deletions	12419*	28.75%	1–2x LoD	83	83	100	96.46
19	Deletions	12422	7.85%	2–3x LoD	94	94	100	96.86
19	Deletions	13551*	11.12%	2–3x LoD	95	95	100	96.90
21	L858R	6224	5.77%	LoD	92	92	100	96.80
20	T790M‡	6240	34.02%	1–2x LoD	94	94	100	96.86
21	L861QΔ	6213	9.24%	LoD	84	83	98.81	94.48
18	G719AΔ	6239	32.50%	LoD	78	77	98.72	94.06
20	S768IΔ	6241*	11.57%	1–2x LoD	82	82	100	96.41
20	Insertions‡	12377*	10.45%	2–3x LoD	93	93	100	96.83
20	Insertions‡	12378*	19.96%	LoD	92	92	100	96.8
–	Wild-type	–	N/A§	–	84	84	100	96.5

* Reproducibility for these mutations was conducted using FFPE cell lines.

† Control C_T for deletion 12370 at LoD dropped out of the working range and no data could be generated at one site (n = 32). The missing data from this site for this mutation was not retested due to lack of sample availability.

‡ Safety and efficacy of GILOTRIF (afatinib) and IRESSA (gefitinib) has not been established for patients with these EGFR mutations. Refer to GILOTRIF or IRESSA drug labeling for more details.

Δ Safety and efficacy of IRESSA (gefitinib) and VIZIMPRO (dacomitinib) has not been established for patients with these EGFR mutations. Refer to IRESSA drug labeling for more details.

§ NA: not applicable.

** See note (**) under Table 1.

Clinical Performance

Correlation to Reference Method using Phase 3 Samples

To demonstrate the accuracy of the *therascreen* EGFR RGQ PCR Kit relative to bi-directional Sanger sequencing, an accuracy study was conducted with clinical trial specimens from the Phase 3 study, 1200.32 clinical trial. This was a blinded study using FFPE clinical specimens from patients in the Phase 3 study, 1200.32 clinical trial. The baseline clinical and demographic characteristics of the patients whose specimens were available for this retrospective testing were comparable to those of otherwise eligible patients whose specimens were not available for retesting. EGFR testing was performed on DNA samples extracted from 360 specimens with bi-directional Sanger sequencing results for exons 18, 19, 20, and 21 that had been blinded to the operators.

Samples with both Sanger and *therascreen* EGFR RGQ PCR Kit valid results were analyzed to assess the OPA, PPA, and NPA based on agreement between the two methods for overall mutation status. These percentages, together with the corresponding two-sided 95% confidence intervals (CI) are summarized in Table 17.

Table 17. *therascreen* EGFR RGQ PCR Kit vs. Sanger concordance in Phase 3 specimens

Measure of agreement	Percent agreement % (N)	95% CI
Positive percent agreement (PPA)	99.4% (157/158)	96.5, 100.0
Negative percent agreement (NPA)	86.6% (175/202)	81.2, 91.0
Overall percent agreement (OPA)	92.2% (332/360)	89.0, 94.8

For the 28 overall mutation status discordant results, 1 (3.6%) sample gave wild-type (i.e., no mutation detected) results by the *therascreen* EGFR RGQ PCR Kit and gave mutation detected results by Sanger sequencing while 27 (96.4%) samples gave mutation detected results by the *therascreen* EGFR RGQ PCR Kit and gave wild-type results by Sanger sequencing.

Clinical Outcome Data – GILOTRIF – to support clinical benefit in NSCLC positive for EGFR Del19 and L858R mutations

The 1200.32 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 non-small cell lung cancer (NSCLC) harboring an EGFR mutation (ClinicalTrials.gov number NCT00949650, also known as 'LUX-Lung 3'). The eligibility of patients for enrollment onto 1200.32 clinical trial was determined by testing the mutation status of NSCLC patients' EGFR status using the Clinical Trial Assay (CTA). Retrospective testing of tissue specimens from patients screened for the 1200.32 clinical trial was performed using the *therascreen* EGFR RGQ PCR Kit. A bridging study was conducted to assess the concordance of the *therascreen* EGFR RGQ PCR Kit with the CTA used to select patients for the 1200.32 clinical trial. The trial objective was to compare the efficacy and safety of afatinib monotherapy with chemotherapy as first-line treatment for the NSCLC patients whose tumors harbor EGFR mutations (i.e., exon 19 deletions, exon 21 L858R substitution, and "other" EGFR mutations). 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 (178 randomized to afatinib and 86 patients randomized to chemotherapy) were tested retrospectively by the companion diagnostic *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 both the overall CTA+ population and the *therascreen* EGFR RGQ PCR Kit+/CTA+ population. The overall efficacy results are summarized in Table 18 and Figure 21.

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 vs. 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).

Table 18. Clinical benefit of patients tested with the *therascreen* EGFR RGQ PCR Kit in the 1200.32 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	

CI: confidence interval; **CTA**: Clinical trial assay.

* Stratified by EGFR mutation status and race.

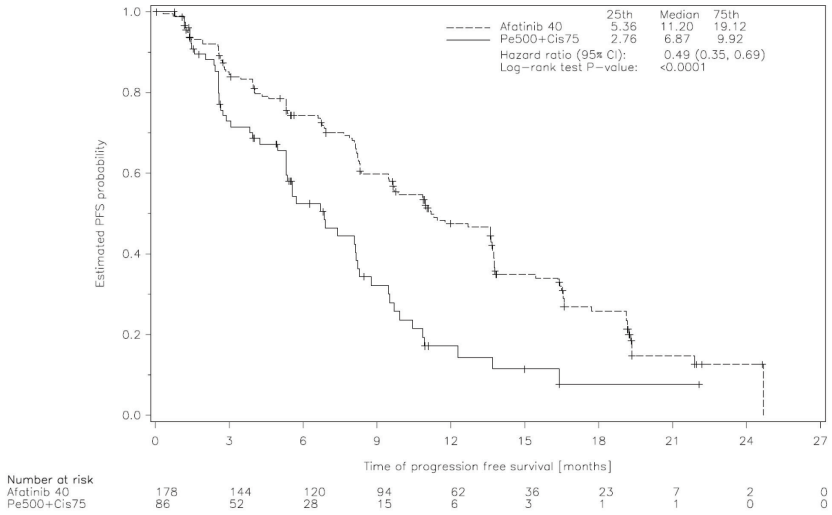

Figure 21. Kaplan-Meier curve of PFS by independent review by treatment group (*therascreen* EGFR RGQ PCR Kit+/CTA+ population).

Given that the *therascreen* EGFR RGQ PCR Kit was not used to select patients for the 1200.32 clinical trial, additional efficacy analyses were conducted to consider patients who were not included in the 1200.32 clinical trial because they were tested negative by the CTA but could have been tested positive by the *therascreen* EGFR RGQ PCR Kit (i.e., *therascreen* EGFR RGQ PCR Kit+/CTA–). In the hypothetical scenario, patients who are *therascreen* EGFR RGQ PCR Kit+/CTA– in the treatment arm (afatinib) were assigned as PFS events at the baseline (Day 1); patients who are *therascreen* EGFR RGQ PCR Kit+/CTA– in the control arm (chemotherapy) were censored for PFS at Month 11 (Day 335, the median PFS for afatinib). Results from this hypothetical scenario showed median PFS times (95% CI) were 11.0 (8.3, 13.6) for the afatinib arm and 6.9 (5.3, 8.8) for the chemotherapy arm, with a hazard ratio (95% CI) of 0.56 (0.39, 0.79) and corresponding p-value of 0.0009. Results from all of the hypothetical analyses were generally similar to those from the primary efficacy analysis.

Clinical Outcome Data – GILOTRIF – to support clinical benefit in NSCLC positive for EGFR L861Q, G719X and S768I mutations

The efficacy of GILOTRIF in patients with NSCLC harboring EGFR mutations, L861Q, G719X, or S768I was evaluated in a pooled analysis of such patients in one of 3 clinical trials. The objective was to identify which of the patients whose tumors have EGFR mutations that may derive a benefit from afatinib treatment.

The following table shows the duration of response to GILOTRIF in patients harboring the L861, G719X or S768I, EGFR mutations, alone or in combination with other secondary mutations. Of the 32 patients treated, 21 demonstrated a response to afatinib. The response duration ranged from 2.8 to 37.3 months depending on the specific mutation or combination of mutations.

Table 19. IRC-assessed responses in patients with NSCLC harboring EGFR mutations L861Q, G719X, and/or S768I from the 1200.22, 1200.32 and 1200.34 trials

EGFR mutation	Number of GILOTRIF-treated patients (N = 32)	Number of confirmed responses (N = 21)	Duration of response (months (N = 21)
S768I	1	1	37.3
S768I and G719X	5	4	4.1, 13.2, 15.2, 29.5+
S768I and L858R	2	1	34.5+
G719X	8	6	5.7+, 8.1, 9.6, 23.5+, 25.2, 31.8+
G719X and L861Q	3	2	2.8+, 6.8
L861Q	12	7	2.8, 4.0, 4.1, 8.3+, 12.9, 15.2, 20.6
L861Q and Del19	1	0	NA

+: Response ongoing at time of assessment.

NA: Not available.

In conclusion, patients with NSCLC harboring the EGFR mutations L861Q, G719X, or S768I demonstrated clinical benefit from afatinib. For those patients with high unmet medical need, afatinib is a suitable treatment option.

Clinical Outcome Data – IRESSA

The IRESSA Follow-up Measure (IFUM) trial was a Phase-4, open-label, single-arm study (NCT01203917) to characterize the efficacy and safety/tolerability of first-line gefitinib in Caucasian patients with stage IIIA/B/IV, EGFR mutation-positive locally advanced or metastatic NSCLC. The IFUM study was designed to evaluate the objective response rate by RECIST criteria in prospectively selected EGFR mutant NSCLC Caucasian patients.

Eligible patients were required to have a deletion in EGFR exon 19, L858R, L861Q, or G719X substitution mutation and no T790M or S768I mutation or exon 20 insertions in tumor specimens as prospectively determined by the Clinical Trial Assay (CTA). Retrospective testing of specimens from patients screened for IFUM clinical trial was performed using the companion diagnostic *therascreen* EGFR RGQ PCR Kit. A bridging study was conducted to assess the concordance of the *therascreen* EGFR RGQ PCR Kit with the CTA used to select patients for

the IFUM clinical trial. The overall concordance between the two assays for detecting EGFR exon 19 deletions and L858R mutation was 98.2% (n = 700/713; 95% CI: 96.9%, 99.0%) with the PPA of 88.2% (n = 90/102; 95%CI: 80.4%, 93.8% and the NPA of 99.8% (n = 610/611; 95% CI: 99.1%, 100.0%).

CTA test results were obtained for 859 screened patients, of which 106 patients were eligible for treatment with gefitinib. Of 859 samples with a CTA result, 765 samples were available for testing retrospectively by the *therascreen* EGFR RGQ PCR Kit, including 87 samples that were EGFR mutation positive by both the CTA and *therascreen* EGFR RGQ PCR Kit.

The major efficacy outcome was objective response rate (ORR) as assessed by a Blinded Independent Central Review (BICR) and investigators. 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.

The overall efficacy results are summarized in Table 19.

Table 19. Clinical benefit of patients tested with the *therascreen* EGFR RGQ PCR Kit in the IFUM clinical trial population

Parameter	*therascreen* EGFR RGQ PCR Kit+ population, n = 87	CTA+ population, n = 106
Objective response rate (ORR) by BICR		
Number of responses (N)	42	53
ORR (%) [95% CI]	48.3 [38.1–58.6]	50.0 [40.6–59.4]
Median duration of response (months)	6.9 [5.6–11.4]	6.0 [5.6–11.1]
Objective response rate (ORR) by investigators		
Number of responses (N)	62	74
ORR (%) [95% CI]	71.3 [61.0–79.7]	69.8 [60.5–77.7]
Median duration of response (months)	8.3 [7.2–11.3]	8.3 [7.6–11.3]

BICR: Blinded independent central review; **CI**: confidence interval; **CTA**: Clinical trial assay.

Note: Kit+ are results positive for exon 19 deletions/L8585R/L861Q/G719X.

Given that the *therascreen* EGFR RGQ PCR Kit was not used to select patients for the IFUM clinical trial, additional efficacy analyses were conducted to consider patients who were not

included in the trial because they were tested negative by the CTA but could have been tested positive by the *therascreen* EGFR RGQ PCR Kit (i.e., *therascreen* EGFR RGQ PCR Kit+/CTA–), as well as patients who were enrolled in the trial but did not have valid retest results from the *therascreen* EGFR RGQ PCR Kit (i.e., *therascreen* EGFR RGQ PCR Kit unknown/CTA+). Results from all of the hypothetical analyses were generally similar to those from the primary efficacy analysis.

Clinical Outcome Data – VIZIMPRO

The VIZIMPRO Study A7471050 was a multi-national, multi-center, randomized, open-label, Phase 3 clinical study determining the efficacy and safety of first line treatment with dacomitinib in patients with newly diagnosed stage IIIB/IV or recurrent NSCLC. All patients were required to have tumors that tested positive for at least one EGFR-activating mutation, deletions in exon 19 or the L858R substitution mutation in exon 21. It was acceptable for patients with the presence of the exon 20 T790M mutation together with either EGFR-activating mutation to be included in this study. All tumors were histo- and/or cytopathologically consistent with adenocarcinoma or its pathologically accepted variants.

In this Phase 3 study, 452 patients were randomized (1:1) to one of two treatment arms: open-label dacomitinib or open-label gefitinib, both of which were administered orally on a continuous daily basis until disease progression, new anticancer therapy instituted, intolerable toxicities were noted, withdrawal of consent, death, or investigator decision dictated by protocol compliance. The randomization was stratified by two factors: race (Japanese versus mainland Chinese versus other East Asian versus non-East Asian, as stated by the patient), and EGFR mutation status (exon 19 deletion mutation versus the L858R substitution mutation in exon 21).

The bridging study re-tested samples from patients who were screened and randomized in Study A7471050. Re-testing determined the mutation status using the CDx for patient specimens collected as part of Study A7471050, in order that this new data could be

compared to the mutation status generated using the CTA for concordance, and to determine the clinical efficacy of the CDx.

Of the 452 patients randomized, 288 cases (64% of the study intent-to-treat [ITT] population, including 142 patients from the dacomitinib arm and 146 patients from the gefitinib arm) were retested retrospectively by the *therascreen* EGFR RGQ PCR Kit and determined to be CDx +ve. Analysis of the 288-patient subset demonstrated that those patients on the dacomitinib arm have 47.9% lower risk of progressive disease or death (HR=0.52, 95 % CI [0.39; 0.70], p<0.0001) than patients on the gefitinib arm.

In the primary analysis, estimating the dacomitinib treatment benefit when using the CDx and accounting for discordant and missing results, the HR was 0.54, 95% CI (0.42, 0.68), p<0.0001. This was similar to the results for the ITT population in Study A7471050 [HR 0.59, 95% CI (0.47, 0.74)]. Further sensitivity analyses assessing the impact of discordant and missing EGFR Kit data on PFS showed consistent results. The sensitivity analysis demonstrated that the results were robust to the adjustments for population prevalence and for the worst-case imputation scenario.

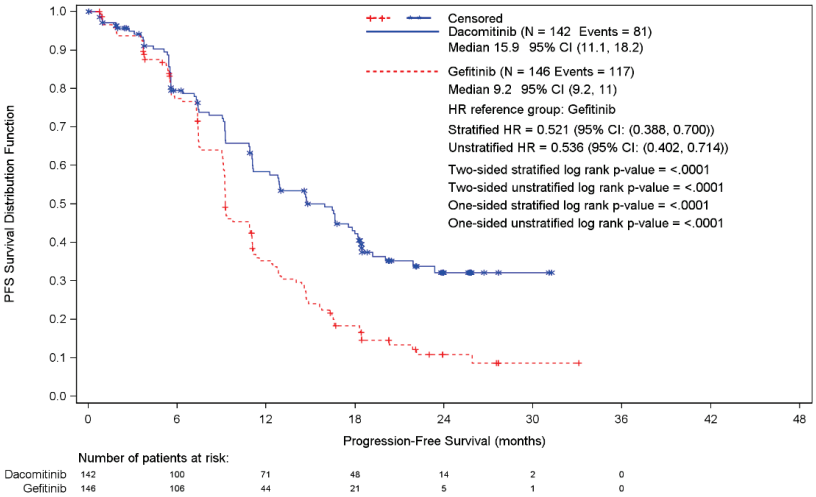

Figure 22: Kaplan-Meier plot of PFS per IRC review: CDx +ve patients in the ITT population.

To evaluate concordance with respect to selection of EGFR-activating mutation positive patients between the CTA and the CDx the PPA, NPA, and OPA, along with the respective two-sided Clopper-Pearson exact 95% confidence interval, using the CDx as the reference, the PPA was 99.7%, 95% CI (98.3%, 100.0%) and the NPA was 84.2%, 95% CI (74.4%, 91.3%). The OPA was 96.5%, 95% CI (94.2%, 98.1%). This indicates a high level of agreement (>99%) for the PPA for the CTA using the CDx as reference.

Using the CTA as a reference, analyzing the CDx evaluable subset, the PPA was 96.1%, 95% CI (93.4%, 97.9%), NPA was 98.6%, 95% CI (92.3%, 100.0%) and OPA was 96.5%, 95% CI (94.2%, 98.1%). Therefore, using the CTA as a reference, the concordance with the CDx was >95% for both PPA and NPA.

There were 14 discordant results between the laboratory CTA result and the CDx result. Thirteen (13) of the patients were CTA positive and were randomized to the study; All 13 patients were CDx negative. One patient was CTA negative and this patient was CDx positive.

References

1. Pao, W., Miller, V.A. (2005) Epidermal growth factor receptor mutations, small molecule kinase inhibitors, and non-small-cell lung cancer: current knowledge and future directions. J. Clin. Oncol. **23**, 2556.

2. Johnson, B.E., Jaenne, P.A. (2005) Epidermal growth factor receptor mutations in patients with non-small cell lung cancer. Cancer Res. **65**, 7525.

3. Inoue, A., Suzuki. T., Fukuhara, T., Maemondo, M., Kimura, Y. (2006) Prospective Phase II study of gefitinib for chemotherapy-naive patients with advanced non-small cell lung cancer with epidermal growth factor receptor gene mutations. J. Clin. Oncol. **24**, 3340.

4. Asahina, H., et al. (2006) A Phase II study of gefitinib as a first-line therapy for advanced non-small cell lung cancers with epidermal growth factor receptor (EGFR) gene mutations. 42nd Ann Mtg of the American Society of Clinical Oncology (ASCO), Atlanta 2-6 June 2006. J. Clin. Oncol. **24(18S)(Suppl)**, Abstr 13014.

5. Paz-Ares, L. et al. A prospective phase II trial of erlotinib in advanced non-small cell lung cancer (NSCLC) patients (p) with mutations in the tyrosine kinase (TK) domain of the epidermal growth factor receptor (EGFR). 42nd Ann Mtg of the American Society of Clinical Oncology (ASCO), Atlanta 2-6 June 2006. J. Clin. Oncol. **24(18S)(Suppl)**, Abstr 7020.

6. Kobayashi, K., et al. (2008) First-line gefitinib for poor PS patients with EGFR mutations. 44th Ann Mtg of the American Society of Clinical Oncology (ASCO), Chicago 31 May-3 June 2008. J. Clin. Oncol. **26(15S)(Suppl)**, Abstr 8070.

7. Sequist, L.V., et al. (2008) First-line gefitinib in patients with advanced non-small cell lung cancer harbouring somatic EGFR mutations. J. Clin. Oncol. **15**, 2442.

8. Porta, R. et al. (2008) Erlotinib customization based on epidermal growth factor receptor (EGFR) mutations in stage IV non-small-cell lung cancer (NSCLC) patients (p). J. Clin. Oncol. **26(May 20 suppl)**, abstr 8038.

9. Jaene, P.A., Johnson, B.E. (2006) Effect of epidermal growth factor receptor tyrosine kinase domain mutation on the outcome of patients with non-small cell lung cancer treated with epidermal growth factor receptor tyrosine kinase inhibitors. 3rd Cambridge Conference on Novel Agents in the Treatment of Lung Cancer: Advances in EGFR-Targeted Agents, Cambridge, 23–24 Sep 2005. Clin. Cancer Res. **12(Suppl.14)**, 4416.

10. Whitcombe, D. et al. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotech. **17**, 804.

11. Thelwell, N. et al. (2000) Mode of Action and Application of Scorpion Primers to Mutation Detection. Nucleic Acids Res. **28**, 3752.

12. Walsh, K., et al. (2014) A cautionary lesson on the use of targeted methods for EGFR mutation analysis: a case report. J. Clin. Pathol. **67**, 734.

13. Huang, J., Wang, Y., Zhai, Y., and Wang, J. (2018) Non-small cell lung cancer harboring a rare EGFR L747P mutation showing intrinsic resistance to both gefitinib and osimertinib (AZD9291): A case report. Thorac. Cancer. **9**, 745.

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/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

		Comments and suggestions
Invalid results		
a)	The storage conditions for one or more components did not comply with the instructions given in "Reagent Storage and Handling,".	Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit, if necessary.
b)	The *therascreen* EGFR RGQ PCR Kit has expired.	Check the storage conditions and the expiration date (see the kit label) of the reagents and, if necessary, use a new *therascreen* EGFR RGQ PCR Kit.
NTC samples show positive results in the FAM channel		
	Contamination occurred during preparation of the PCR	Repeat the PCR with new reagents. 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.

Rotor-Gene Q *therascreen* EGFR Assay Package flags

Table 20 lists the possible flags that may be generated by the Rotor-Gene Q *therascreen* EGFR Assay Package, their meaning, and actions to be taken.

The 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 20. Rotor-Gene Q *therascreen* EGFR Assay Package flags

Flag	Meaning	Action to be taken
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 paying close attention to mixing steps.
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 paying close attention to mixing steps.
NTC_INVALID_DATA	PCR run invalid — fluorescence data in negative control cannot be interpreted.	Repeat the entire PCR run paying close attention to mixing steps.
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) paying close attention to mixing steps.
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 EGFR mutation detection run to repeat sample. Or if the sample has been diluted following the DNA sample assessment run, proceed straight to EGFR mutation detection run with diluted sample.

Table continued on next page

Table continued from previous page

Flag	Meaning	Action to be taken
SAMPLE_CTRL_FAIL	Sample invalid — FAM C_T too high in sample control reaction.	Set up new PCR run to repeat sample.
If the quantity of DNA is still insufficient, extract two further FFPE tissue sections, if available. Set up a new PCR run to test this purification. If sample invalid, repeat the PCR run on the second purification. 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.	For samples that also generate a SAMPLE_POSITIVE_ AND_INVALID flag with a mutation detected (or not detected) in a clinically relevant mutation reaction mix – report results, no further testing required.
If sample is given invalid status, dilute the remaining sample with the water supplied with the kit using the assumption that diluting 1:1 will increase the C_T of the control reaction by 1.0, ensuring the final volume is >40 µl (e.g., 40 µl DNA and 40 µl water from the tube marked DIL). Set up new PCR run to repeat sample. If invalid on the repeat PCR run, extract the sample from two further FFPE sections. Set up a new PCR run to test the fresh purifications. If the second purification is invalid, dilute as described above. 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.		

Table continued on next page

Table continued from previous page

Flag	Meaning	Action to be taken
SAMPLE_POSITIVE _AND _INVALID	One or more mutations for a sample are positive at the same time one or more mutations for the same sample are invalid.	For samples that generate a SAMPLE POSITIVE AND INVALID flag with an INVALID result obtained in a clinically relevant mutation reaction mix - retest the sample with all reaction mixes following the specific invalid flag action.
If a SAMPLE_INT_CTRL_FAIL flag is generated in combination with another flag for the affected sample, then the action of diluting the sample from the SAMPLE_INT_CTRL_FAIL flag must be followed. Setup a new PCR run and retest the sample. For samples that generate a SAMPLE POSITIVE AND INVALID flag with an INVALID result obtained in a clinically relevant mutation reaction mix on the repeat PCR run - extract the sample from two further FFPE sections. Set up a new PCR run with all reaction mixes to test this purification. If this sample produces an invalid result again for a clinically relevant mutation reaction mix - repeat the sample with all reaction mixes following the specific invalid flag action. If SAMPLE_INT_CTRL_FAIL is generated in combination with another flag for the affected sample, then the action of diluting the sample from the SAMPLE_INT_CTRL_FAIL flag must be followed. Setup a new PCR run and retest this sample. If SAMPLE_POS_AND_INVALID flag is observed upon this repeat, the sample is given an indeterminate mutation status.		
SAMPLE_INT_CTRL _EARLY_CT	Mutation tube invalid — C_T HEX too low for sample (internal control)	For samples that also generate a SAMPLE_POSITIVE_AND_ INVALID flag with a mutation detected (or not detected) in a clinically relevant mutation reaction mix – report results, no further testing required.
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 purification. If invalid, repeat the PCR run on the second purification. 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 cannot be interpreted.	For samples that also generate a SAMPLE_POSITIVE_AND_ INVALID flag with a mutation detected (or not detected) in a clinically relevant mutation reaction mix – report results, no further testing required.
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 purification. If invalid, repeat the PCR run on the second purification. 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.		
MUTATION_EARLY_CT	Sample invalid — Delta C_T too low or C_T is below cutoff range	Set up new PCR run to repeat sample paying close attention to mixing samples.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
 <N>	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number
	Components
	Contains
	Number
	Global Trade Item Number
Rn	R is for revision of the Instructions for Use (Handbook) and n is the revision number
	Temperature limitation
	Manufacturer

Symbol	Symbol definition
	Consult instructions for use
	Keep away from sunlight
	Prescription Use Only

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 one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Appendix: Installation of the Rotor-Gene Q *therascreen* EGFR Assay Package

The *therascreen* EGFR RGQ PCR Kit is designed for use with the Rotor-Gene Q MDx instrument with a 72-well rotor. The Rotor-Gene Q *therascreen* EGFR Assay Package is available for download in the *therascreen* EGFR RGQ PCR Kit product webpage at **www.qiagen.com.** Go to **Product Resources** > **Supplementary Protocols** to download the assay package.

The package includes "*therascreen* EGFR Control Run Locked Template" and "*therascreen* EGFR Locked Template".

The printable documentation templates and PCR layouts can be downloaded in the *therascreen* EGFR RGQ PCR Kit product webpage at **www.qiagen.com**. Go to **Product Resources** > **Instrument Technical Documents** to download and print the documents. The documentation templates and PCR layouts can be used to record FFPE sample preparation using the QIAamp DSP DNA FFPE Tissue Kit, DNA sample assessment using the *therascreen* EGFR RGQ PCR Kit, and detection of EGFR mutations using the *therascreen* EGFR RGQ PCR Kit.

Note: The Rotor-Gene Q *therascreen* EGFR Assay Package only works with Rotor-Gene Q Software version 2.3.5. Make sure that the correct version of Rotor-Gene Q software is installed before proceeding with the Rotor-Gene Q *therascreen* EGFR Assay Package installation.

Procedure

1. Download the Rotor-Gene Q *therascreen* EGFR Assay Package from **www.qiagen.com** and transfer it to a virus-free USB storage device.

   Note: The assay package is available in the *therascreen* EGFR RGQ PCR Kit product webpage. Go to **Product Resources** > **Supplementary Protocols** to download the assay package.

2. Insert the USB storage device into the laptop connected to the Rotor-Gene Q MDx instrument.
3. Locate the **Rotor-Gene Q *therascreen* EGFR Assay Package** file.
4. Right-click **Rotor-Gene Q *therascreen* EGFR Assay Package** then select **Extract all** to unzip the file.
5. Double-click **therascreen_EGFR_Assay_Package_3.1.2.exe** to start the installation.

   The setup wizard will appear.
6. Click **Next** to continue (Figure 23).

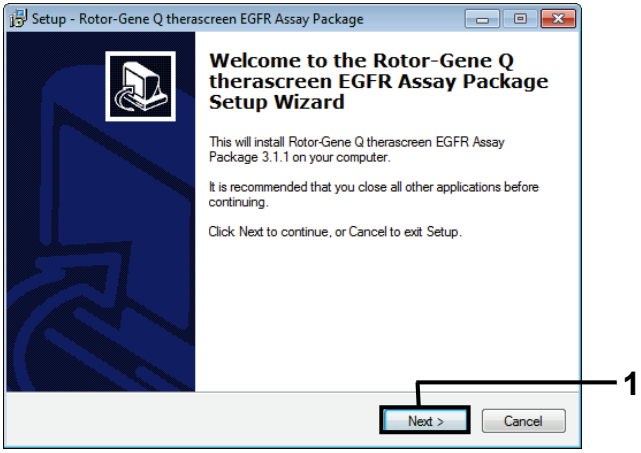

Figure 23. The "Setup" dialog box. 1 = "Next".

7. Read the License Agreement in the "License Agreement" dialog box and check **I accept the agreement**. Click **Next** to continue (Figure 24).

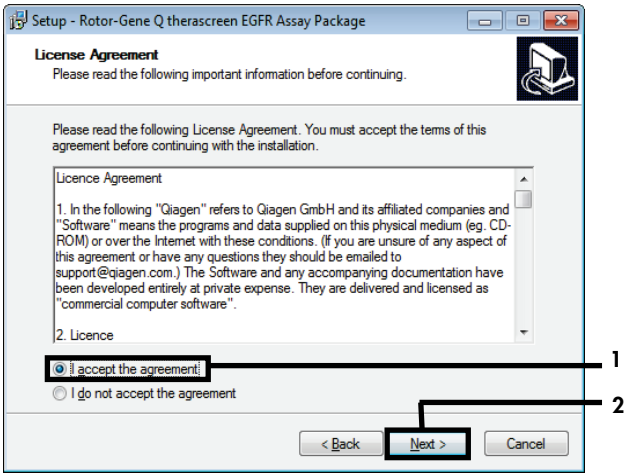

Figure 24. The "License Agreement" dialog box. 1 = "I accept the agreement" statement, 2 = "Next".

The template setup will start automatically and a final "Setup" dialog box will appear.

8. Click **Finish** to exit the setup wizard (Figure 25).

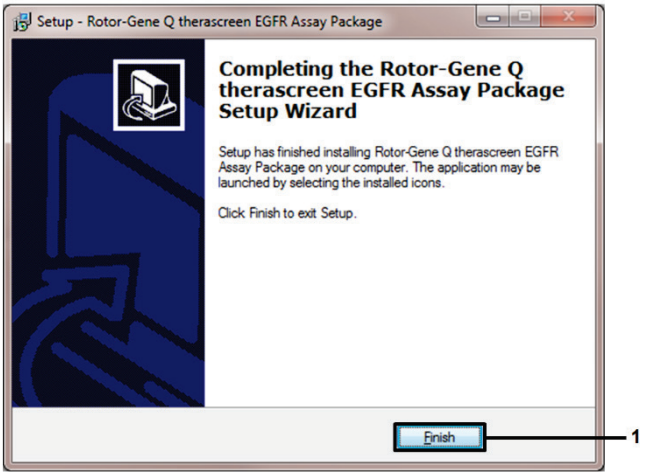

Figure 25. Completing the setup wizard. 1 = "Finish".

9. Restart the computer. Shortcuts to both the "*therascreen* EGFR Control Run Locked Template" and "*therascreen* EGFR Locked Template" will be generated automatically and appear on the desktop.

Ordering Information

Product	Contents	Cat. no.
therascreen EGFR RGQ PCR Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, Water, *Taq* DNA Polymerase	870121
Rotor-Gene Q *therascreen* EGFR Assay Package	Software protocol package for use with the *therascreen* EGFR RGQ PCR Kit and the QIAGEN Rotor-Gene Q MDx instrument	Download
Rotor-Gene Q MDx and accessories		
Rotor-Gene Q MDx Platform (US)	Real-time PCR cycler, laptop computer, software, accessories, 1-year warranty on parts and labor	9002035
Rotor-Gene Q MDx System (US)	Real-time PCR cycler, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002036
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction set up 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
Related products		
QIAamp DSP DNA FFPE Tissue Kit (50)	For 50 DNA preps: QIAamp MinElute® Columns, Proteinase K, Buffers, and Collection Tubes (2 ml)	60404

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

Document Revision History

Date	Changes
R8, October 2019	Updated limitations of procedure statement and analytical specificity section with information on the Exon 19 deletions assay and additional mutations that have been found to cross-react with the therascreen EGFR RGQ PCR Kit.
R9, November 2019	Change in legal manufacturer (cover page)
R10, June 2020	Updated version number of EGFR assay package from 3.1.1 to 3.1.2 Updated the references to RGQ software version from 2.3 to 2.3.5 or later Updated Table 12 to implement new cutoff range for the delta C_T (ΔC_T) Moved Table 13 from Analytical Sensitivity – Upper cutoff values section to Analytical sensitivity – Limit of Detection section Updated all Protocols chapters to include information about the importance of mixing in Important points before starting sections; highlighted the mixing details at all mixing steps of the protocols; Added mixing steps where required Added MUTATION_CT_EARLY flag in Table 20 Removed all references to *therascreen* EGFR Assay Package CDs and replaced with download information

Page left intentionally blank

Page left intentionally blank

Limited License Agreement for *therascreen* EGFR RGQ PCR Kit

Use of this product signifies the agreement of any purchaser or user of the product to the following terms:

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this handbook, and additional protocols available at **www.qiagen.com**. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.

2. Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.

3. This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.

4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.

5. The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the panel and/or its components.

For updated license terms, see **www.qiagen.com**.