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therascreen[®] EGFR Plus RGQ PCR Kit Instructions for Use



Version 1



For In Vitro Diagnostic Use

For use with plasma or FFPE tissue

For use with Rotor-Gene[®] Q MDx 5plex HRM instrument and Rotor-Gene[®] AssayManager[®]



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Intended Use

The *therascreen* EGFR Plus RGQ PCR Kit is an *in vitro* diagnostic real-time PCR test for the qualitative detection and identification of mutations in exons 18, 19, 20, and 21 of the epidermal growth factor receptor (*EGFR*) gene (1) in DNA derived from formalin-fixed paraffin-embedded (FFPE) tumor tissue and plasma from non-small cell lung cancer (NSCLC) patients.

The *therascreen* EGFR Plus RGQ PCR Kit is further indicated for the semi-quantitative measurement of mutations in exons 18, 20, and 21 of the *EGFR* gene of human plasma as an aid in the management of NSCLC cancer patients.

The test is to be used by trained personnel in a professional laboratory environment

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

Indication for use statement

The test is intended to be used as an aid in selecting patients with NSCLC for therapy with an *EGFR* tyrosine kinase inhibitor (TKI).

Description and Principle

Summary and explanation

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

The *therascreen* EGFR Plus RGQ PCR Kit is a real-time PCR (polymerase chain reaction) test for the detection of 42 mutations in the *EGFR* cancer-related gene using ARMS (Amplification Refractory Mutation System) (10, 11) and PCR clamp technologies for the qualitative detection and identification of mutations in the *EGFR* gene; exons 18, 19, 20, and 21 (Table 1). The kit allows the semi-quantification of G719X (X = A, S, or C; exon 18), T790M (exon 20), C797Sa and C797Sb (exon 20), S768I (exon 20), L858R (exon 21), and L861Q (exon 21) in DNA samples extracted from human plasma. In summary:

- G719X in exon 18 (detects and semi-quantifies G719S, G719A, or G719C, but does not distinguish between them)
- 28 deletions in exon 19 (detects the presence of any of the 28 deletions but does not distinguish between them)
- S768I, T790M, C797Sa and C797Sb in exon 20 (detects and semi-quantifies all four mutations, but does not distinguish between C797Sa and C797Sb)
- Five insertions in exon 20 (detects the presence of any of the five insertions, but does not distinguish between them)

The methods used are highly selective and, depending on the total amount of DNA present, enable detection of a low percentage of mutant DNA in a background of wild-type genomic

DNA. These selectivity and detection limits are superior to technologies such as dye-terminator sequencing.

Table 1. List of mutations and COSMIC identities

Exon	Mutation	COSMIC ID*	Base change
18	G719A	6239	c.2156G>C
	G719S	6252	c.2155G>A
	G719C	6253	c.2155G>T
19	Deletions	26038	c.2233_2247del15
		13550	c.2235_2248>AATTC
		6223	c.2235_2249del15
		6225	c.2236_2250del15
		18427	c.2237_2257>TCT
		6220	c.2238_2255del18
		12367	c.2237_2254del18
		12384	c.2237_2255>T
		12678	c.2237_2251del15
		13551	c.2235_2252>AAT
		13552	c.2235_2251>AATTC
		12386	c.2237_2252>T
		12416	c.2237_2253>TTGCT
		12728	c.2236_2253del18
		12422	c.2238_2248>GC
12382	c.2239_2248TTAAGAGAAG>C		

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Table 1. List of mutations and COSMIC identities (cont'd)

Exon	Mutation	COSMIC ID*	Base change
19	Deletions	6218	c.2239_2247delTTAAGAGAA
		12387	c.2239_2258>CA
		12370	c.2240_2257del18
		12403	c.2239_2256>CAA
		6255	c.2239_2256del18
		12383	c.2239_2251>C
		12419	c.2238_2252>GCA
		6210	c.2240_2251del12
		23571	c.2238_2252del15
		12369 [†]	c.2240_2254del15
		13556	c.2253_2276del24
		12385	c.2235_2255>AAT
20	S768I	6241	c.2303G>T
	Insertions	12376	c.2307_2308insGCCAGCGTG
		12378	c.2310_2311insGGT
		12377	c.2319_2320insCAC
		13428	c.2311_2312insGCGTGGACA
		13558	c.2309_2310AC>CCAGCGTGGAT
	T790M	6240	c.2369C>T
	C797Sa	6493937	c.2389T>A
C797Sb	5945664	c.2390G>C	

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Table 1. List of mutations and COSMIC identities (cont'd)

Exon	Mutation	COSMIC ID*	Base change
21	L858R	6224	c.2573T>G
	L861Q	6213	c.2582T>A

* COSMIC: Catalogue Of Somatic Mutations In Cancer: <http://cancer.sanger.ac.uk/cosmic>.

† According to the new COSMIC database, due to the similarities in sequence after the deletion has occurred, deletion 6254 is combined with deletion 12369.

Principle of the procedure

The *therascreen* EGFR Plus RGQ PCR Kit uses real-time PCR for the detection of 42 mutations in the *EGFR* gene (exons 18, 19, 20, and 21) and the semi-quantification of G719X (with X = A, S or C; exon 18), T790M (exon 20), C797Sa and C797Sb (exon 20), S768I (exon 20), L858R (exon 21) and L861Q (exon 21) in DNA samples extracted from human plasma. The *therascreen* EGFR Plus RGQ PCR Kit will test genomic DNA (gDNA) extracted from FFPE tumor tissue and circulating cell-free DNA (ccfDNA) extracted from plasma samples from non-small cell lung cancer (NSCLC) patients. EGFR mutation status and semi-quantification (when applicable) of pure ccfDNA are determined using the *therascreen* EGFR RGQ PCR Kit on Rotor-Gene Q MDx 5plex HRM instrument performed with the Rotor-Gene AssayManager (RGAM) software version 2.1 (or higher) combined with Gamma plug-in version 1.0.0 (or higher) associated to the plasma dedicated Assay Profile. Data analysis and result interpretation are fully automated and managed by RGAM (Figure 1).

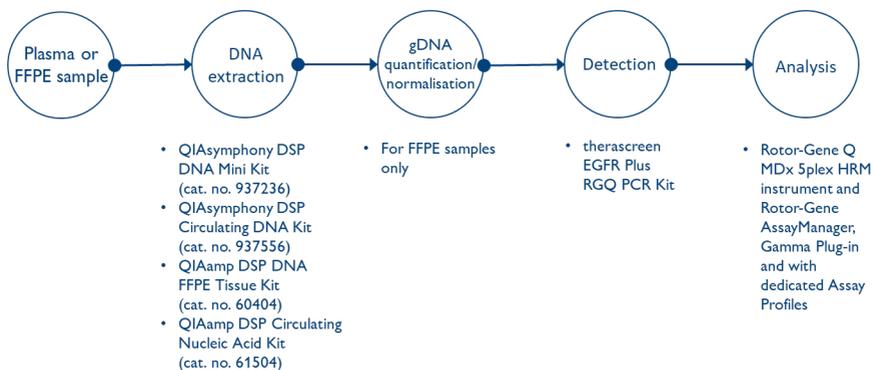


Figure 1. *therascreen* EGFR Plus RGQ PCR Kit workflow.

Technology

The use of qPCR permits the accurate detection of PCR products during the exponential phase of the PCR amplification process. qPCR data can be rapidly obtained, without post-PCR processing, by real-time detection of fluorescent signals during PCR cycling.

The *therascreen* EGFR Plus RGQ PCR assays exploit the qPCR oligonucleotide hydrolysis principle. During PCR, forward and reverse primers hybridize to a specific sequence. Another dye-linked oligonucleotide is contained in the same mix. This probe, which consists of an oligonucleotide labeled with a 5' reporter dye and a downstream 3' dye-free quencher, hybridizes to a target sequence within the PCR product. qPCR analysis with hydrolysis probes exploits the 5'→3' exonuclease activity of the *Thermus aquaticus* (*Taq*) DNA polymerase. When the probe is intact, the proximity of the reporter dye to the quencher results in suppression of the reporter fluorescence, primarily by Förster-type energy transfer.

During PCR, if the target of interest is present, both forward and reverse primers specifically anneal and flank the probe. The 5'→3' exonuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher only if the 3' oligonucleotides hybridize to the target. The probe fragments are then displaced from the target and polymerization of the strand continues. The 3' end of the probe is blocked to prevent extension of the probe during PCR (Figure 2). This process occurs in every cycle and does not interfere with the exponential accumulation of product.

The increase in fluorescence signal is detected only if the target sequence is complementary to the primers and probe and hence amplified during PCR.

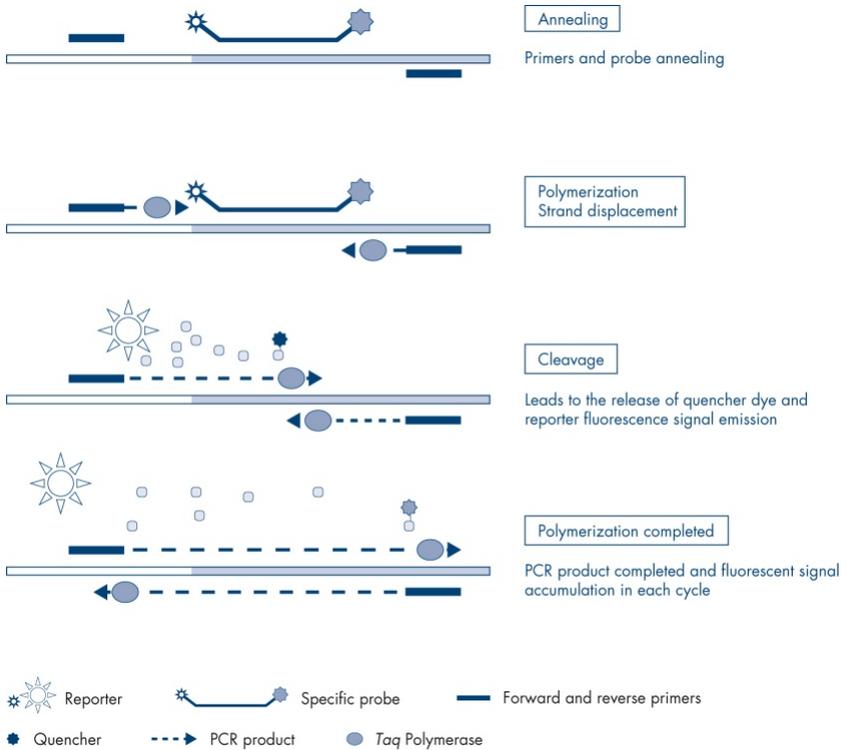


Figure 2. Reaction principle.

In the *therascreen* EGFR Plus RGQ PCR Kit, mutation-specific reactions use ARMS (amplification-refractory mutation system) and clamp designs to detect, identify, and semi-quantify (when applicable) mutations in DNA extracted from plasma.

ARMS

Amplification-refractory mutation system (ARMS) 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 (Figure 3).

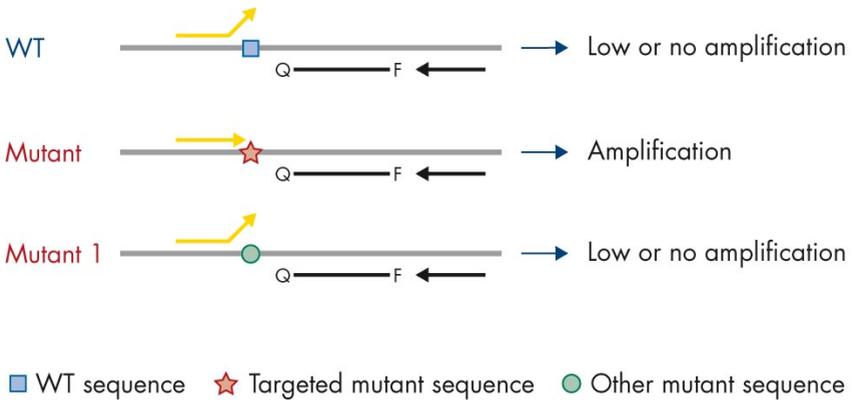


Figure 3. Identification of specific mutation by ARMS PCR. WT: Wild type. Q–F: Double-dye probe. D: Forward and reverse primers.

PCR clamp

This method is used to detect several variants localized in the same hotspot (e.g., *EGFR* deletions in exon 19). The clamp assay combines standard primers and probe with an additional oligonucleotide that is 3'-blocked by the addition of a phosphate group, in order to prevent PCR elongation. The clamp oligonucleotide, as well as the primers and probe, are specific to the wild-type sequence (PCR clamping). When the PCR template contains the wild-type sequence, the clamp hybridizes before the primer, due to a higher T_m leading to no or low amplification. In contrast, when a mutated sequence is present, the clamp cannot bind, which allows primer annealing and amplification (Figure 4).

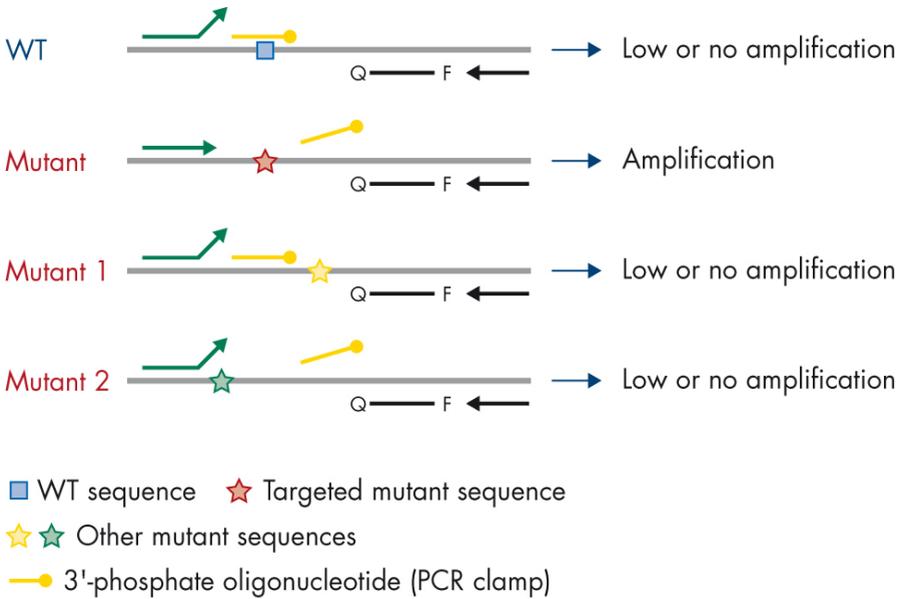


Figure 4. Mutation detection by clamp technology. WT: Wild type. Q–F: Double-dye probe. D: Forward and reverse primers.

Materials Provided

Kit contents

<i>therascreen</i> EGFR Plus RGQ PCR Kit			(24)
Catalog no.			874611
Number of reactions			24
Color	Identity	Tube ID	Volume
Green	T790M & L861Q Mix	<i>EGFR</i> T790M & L861Q Mix	280 μ l
Yellow	Insertions & G719X Mix	<i>EGFR</i> Insertions & G719X Mix	280 μ l
Purple	L858R & C797S Mix	<i>EGFR</i> L858R & C797S Mix	280 μ l
Orange	Deletions & S768I Mix	<i>EGFR</i> Deletions & S768I Mix	280 μ l
Red	<i>EGFR</i> Positive Control	<i>EGFR</i> Positive Control	190 μ l
Blue	PCR Master Mix	<i>EGFR</i> PCR Master Mix	2 \times 940 μ l
Colorless	Water for NTC	NTC	1.9 ml
Colorless	Water for sample dilution	Dil.	1.9 ml

Note: The contents of the *therascreen* EGFR Plus RGQ PCR Kit are sufficient for 24 samples (the kit contains sufficient reagents for up to four qPCR runs of six samples per run, including run controls).

Kit format and assays

Mutation assays

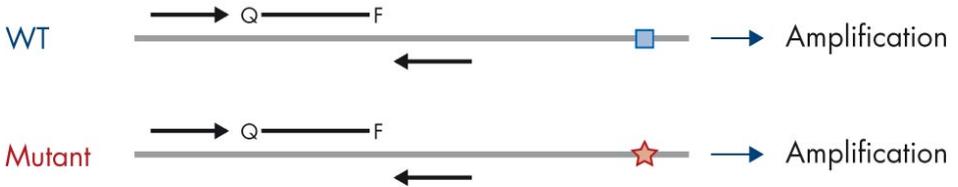
Four primer and probe mixes are supplied in the *therascreen* EGFR Plus RGQ PCR Kit:

- T790M and L861Q
- Insertions (exon 20) and G719X
- L858R and C797S
- Deletions (exon 19) and S768I

All primer and probe mixes, when combined with the PCR Master Mix, allow the detection of targets that are labeled with carboxyfluorescein (FAM™), CAL Fluor® Red 610 and an internal control labeled with hexachlorofluorescein (HEX™).

Internal control assay

The internal amplification control reaction, labeled with HEX, is used to assess the total amplifiable *EGFR* DNA template in a mutated and a non-mutated (wild type) sample (Figure 5), and to identify reaction failures due to suboptimal DNA input, or the presence of inhibitory substances in the sample matrix. This internal amplification reaction amplifies a region of exon 2 of the *EGFR* gene. The primers and probe have been designed to avoid any known *EGFR* polymorphisms.



■ WT sequence ★ Targeted mutant sequence

Figure 5. EGFR exon 2 internal control (IC) detection. WT: Wild-type. Q–F: Double-dye probe. D Forward and reverse primers.

Water for sample dilution (Dil.)

The *therascreen* EGFR Plus RGQ PCR Kit contains Nuclease-Free Water to be used for gDNA sample dilution.

Controls

Each PCR run must contain a positive control (PC) and a negative control (NTC) for each of the four assays.

Positive Control (PC)

Each run must contain a positive control in tubes 1–4. The *therascreen* EGFR Plus RGQ PCR Kit contains one *EGFR* Positive Control (PC) to be used as the template in the positive control reaction. The positive control results will be assessed automatically by the Rotor-Gene AssayManager® to ensure that the kit performs within the predefined acceptance criteria.

No template control (NTC)

Each run must contain a negative control (no template control; NTC) in tubes 5–8. The *therascreen* EGFR Plus RGQ PCR Kit contains water for the NTC to be used as the “template” for the no template control. The no template control is used to assess any potential reagent and environment contamination.

Platform and software

The *therascreen* EGFR Plus RGQ PCR Kit is specifically designed to be used with Rotor-Gene® Q MDx 5plex HRM instruments* with fluorescent channels for cycling green, yellow, and red with the Rotor-Gene AssayManager v2.1.X (X≥0) core software, the Gamma plug-in v1.0.X (X≥0), and the *therascreen* EGFR Plus Assay Profiles.

Two *therascreen* EGFR Assay Profiles are available: the **therascreen EGFR Plus FFPE** (for FFPE sample assessment) and the **therascreen EGFR Plus Plasma** (for Plasma sample assessment). The assay profiles contain the PCR run parameters, and analysis parameters allowing an automated result interpretation.

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

Materials Required but Not Provided

Additional reagents for sample preparation

- Deparaffinization Solution (cat. no. 19093 or 939018) for manual and automated gDNA preparation from FFPE samples
- QIASymphony® DSP DNA Mini Kit (cat. no. 937236) for automated gDNA preparation from FFPE samples
- QIASymphony DSP Circulating DNA Kit (cat. no. 937556) for automated ccfDNA preparation from plasma samples
- QIAamp® DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual gDNA preparation from FFPE samples
- QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual ccfDNA preparation from plasma samples

Note: Materials required but not provided for the DNA extraction kits listed above are detailed in the respective kit handbooks.

- RNase A (cat. no. 19101) for manual or automated gDNA sample preparation from FFPE samples
- Buffer ATL (cat. no. 939016) for the deparaffinization protocol used with the QIASymphony DSP DNA Mini Kit (cat. no. 937236) or QIASymphony DNA Mini Kit (cat. no. 931236)

Consumables and general laboratory equipment

- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR Reaction Mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA

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

- Nuclease-free, aerosol-resistant, sterile PCR pipette tips with hydrophobic filters (pipette tips with aerosol barriers are recommended to help prevent cross-contamination)
- Vortex mixer*
- Benchtop centrifuge* with rotor for 0.5 ml, 1.5 ml and 2.0 ml reaction tubes (capable of attaining 13,000–14,000 rpm)
- DNase, RNase, DNA-free, sterile 1.5 or 2.0 ml microcentrifuge tubes for preparing DNA and PCR reaction mixes
- Strip Tubes and Caps, 0.1 ml, for the Rotor-Gene Q MDx 5plex HRM instrument (cat. no. 981103 or 981106)
- DNA quantification instrument
- Sample tubes (e.g., 2 ml Sarstedt tube [cat. no. 72.693]) for automated gDNA preparation (from FFPE blocks). Compatible primary and secondary tube formats are listed at www.qiagen.com/goto/dspdnakits.
- Single-use, sterile scalpel for manual and automated gDNA preparation (from FFPE section on slide sample)
- Sterile and IVD use phosphate-buffered saline (PBS, may be required for topping up plasma sample volumes)

Equipment

Equipment for automated sample preparation

- QIAasymphony SP instrument* (cat. no. 9001297) and provided accessories
Note: Required accessories are detailed in the respective extraction kit handbooks and in the *QIAasymphony SP/AS User Manual General Description*.
- QIAasymphony software version 4.0 or later

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

- QIASymphony Tissue_LC_200_DSP protocol for automated gDNA preparation from FFPE samples (see www.qiagen.com/shop/sample-technologies/dna/genomic-dna/qiasymphony-dsp-dna-kits-row/#resources)
- QIASymphony circDNA_2000_DSP protocol for automated ccfDNA preparation from plasma samples (see www.qiagen.com/shop/sample-technologies/dna/genomic-dna/qiasymphony-dsp-circulating-dna-kit/#resources)

Equipment and materials for qPCR

- Rotor-Gene Q MDx 5plex HRM instrument* with fluorescence channels for Cycling Green, Cycling Red, and Cycling Yellow (detection of FAM, CAL Fluor Red 610, and HEX, respectively)
- Loading Block 72 × 0.1 ml Tubes, aluminum block for manual reaction setup with a single-channel pipette (cat. no. 9018901)
- 72-Well Rotor (cat. no. 9018903), Locking Ring 72-Well Rotor (cat. no. 9018904) and Rotor Holder (cat. no. 9018908)
- Rotor-Gene AssayManager software version 2.1.x (where x = 1 or later)
- Rotor-Gene AssayManager Gamma Plug-in installed, version 1.0.x (where x = 0 or later)
- EGFR RGQ PCR Assay Profile version 1.0.x (where x = 0 or later)
 - **therascreen_EGFR_Plus_FFPE** for FFPE samples
 - **therascreen_EGFR_Plus_Plasma** for plasma samples

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

Warnings and Precautions

For customers in the European Union, please be aware that you are required to report serious incidents that have occurred in relation to the device to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.

Safety information

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

For safety information regarding the QIA Symphony SP instrument and the Rotor-Gene Q instrument, refer to the user manual supplied with the instrument.

- 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.

Precautions

Use of *therascreen* EGFR Plus RGQ PCR Kit requires good laboratory practices, including traceability, maintenance of equipment dedicated to molecular biology, and compliance with applicable regulations and relevant standards.

This kit is intended for *in vitro* diagnostic use. Reagents and instructions supplied in this kit have been tested for optimal performance.

The user should always pay attention to the following:

- The test is for use with FFPE and plasma NSCLC specimens.
- Use extreme caution to prevent contamination of samples and reagents with *EGFR*-positive material (i.e., Positive Control) or potentially *EGFR*-positive material (i.e., specimens to be tested).
 - Change the scalpels between samples when scraping the tissue.
 - Use separate, dedicated pipettes for DNA extraction/preparation, setting up PCR reaction mixes (pre-PCR reaction mix preparation) and adding DNA template into PCR tubes.
 - Use fresh aerosol-resistant pipette tips for all pipetting steps to avoid cross-contamination of the samples and reagents. Use extreme caution to prevent DNA or PCR product carryover contamination which may result in a false-positive signal.
 - Preparation and dispensing of reaction mixes must be carried out in a dedicated area separate from the DNA preparation area where no DNA matrices (DNA, plasmid or PCR products) are introduced. In this same area, add water in NTC tubes and close them.
 - Add DNA template in a separate zone, preferably in a separate room, with dedicated equipment (pipettes, tips, etc.).
 - Rotor-Gene Q tubes must not be opened after the PCR run has finished. This is to prevent laboratory contamination with post-PCR products.
- *therascreen* EGFR Plus RGQ PCR Kit reagents should be protected from light, temperature, and repeated thawing and freezing, otherwise the kit performance may be altered.
- Frozen components are thawed completely at room temperature (15–25°C) (or in a refrigerator (2–8 °C) and protected from light. Check regularly to see whether the material is already thawed.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.

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- Reagents for the *therascreen* EGFR Plus RGQ PCR Kit have been optimally diluted. Do not dilute reagents further as this may result in a loss of performance.
 - Do not use reaction volumes (reaction mix plus sample) of less than 25 μ l since this will increase the risk of a false-negative result.
 - All reagents supplied in the *therascreen* EGFR Plus RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same *therascreen* EGFR Plus RGQ PCR Kit.
 - Do not substitute the reagents in the *therascreen* EGFR Plus RGQ PCR Kit or between *therascreen* EGFR Plus RGQ PCR Kit lots, as this may affect performance.
 - Do not use expired or incorrectly stored components.
 - Caution must be observed to ensure correct sample testing and analysis with emphasis on eliminating wrong sample entry, loading error, pipetting error, and placing the PCR strip tubes into the appropriate positions of the 72-well rotor.
 - Make sure the samples are handled in a systematic way to ensure correct identification and traceability.
 - Use extreme caution to prevent contamination by DNase, which may cause degradation of the template DNA. Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials) and wear gloves when performing the assay.
 - **Note:** The product is to be used only by experienced laboratory personnel familiar with laboratory procedures and the Rotor-Gene Q MDx 5plex HRM instrument.

Reagent Storage and Handling

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.

Shipping conditions

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

For shipping conditions relating to the DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

Storage conditions

The *therascreen* EGFR Plus RGQ PCR Kit should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light.

Note: All fluorescently labeled probes in the reaction mix reagents are light sensitive. Protect reaction mix reagents from light to avoid photo bleaching.

Repeated freezing and thawing should be avoided. Reagents should ideally be subjected to a maximum of four freeze–thaw cycles.

For storage and handling information relating to the DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

Stability

When stored under the specified storage conditions, the *therascreen* EGFR Plus RGQ PCR Kit is stable until the stated expiration date on the label. Avoid unnecessary freeze–thawing of the contents of the kit.

Once opened, reagents can be stored in their original packaging at -30 to -15°C until the stated expiration date shown on the packaging. The total time prior to run once the PCR reactions are set up should not exceed 24 hours if stored in the refrigerator (2 – 8°C ; this time includes both the PCR setup and storage).

For stability information relating to the DNA extraction kits and associated reagents to be used, refer to the respective kit handbooks.

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.

Sample Storage and Handling

Sample material is human genomic DNA extracted from FFPE tumor tissue or circulating cell-free DNA (ccfDNA) extracted from 2K-EDTA plasma.

Samples must be transported according to standard pathology methodology to ensure specimen quality.

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

Note: For optimal use of the reagents in the *therascreen* EGFR Plus RGQ PCR Kit, samples should be batched. If samples are tested individually, this will use more reagents and reduce the number of samples that can be tested with the kit.

FFPE samples

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

To prepare tissue samples for gDNA extraction:

- Standard formalin fixation and paraffin-embedding procedures should be used. Refer to the relevant extraction kit handbook for more details.
- Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides. Use a trained individual (e.g., a pathologist) to assess a Hematoxylin & Eosin (H&E)-stained section to confirm that there is tumor present. The stained sections must not be used for DNA extraction.
- The starting material for gDNA purification is sections of FFPE tissue (ideally freshly cut).

- Store all FFPE blocks and slides at room temperature (15–25°C). FFPE sections mounted on glass slides may be stored at ambient temperature for up to 1 month prior to DNA extraction.

Plasma samples

Use standard laboratory procedures to prepare plasma from 2K-EDTA whole blood samples. Refer to the relevant extraction kit handbook for more details.

If fresh plasma is used for nucleic acid extraction on the same day, store at 2–8°C until further processing. For longer-term storage, keep plasma frozen at –30 to –15°C or –90 to –65°C. It is recommended to use aliquots, to avoid freeze–thawing of plasma samples. Repeated freeze–thawing leads to denaturation and precipitation of proteins, which may result in reduced yields of circulating cell-free nucleic acids.

Genomic DNA and circulating cell-free DNA samples

Genomic DNA extracted from FFPE tissue and circulating cell-free DNA extracted from plasma should be stored at 2–8°C for short-term storage (up to 24 hours) and –30 to –15°C (or –90 to –65°C) if long-term storage is required. Avoid unnecessary freeze–thawing of the extracted gDNA and ccfDNA. Frozen eluates must not be thawed more than three times.

Procedure

Protocol: DNA extraction and preparation

Things to do before starting

- Make sure the operator is trained in using the instruments and extraction kits needed for DNA extraction and sample preparation. If required, instrument training can be provided upon installation (see “Ordering Information”, page 110).
- Read the “Materials Required but Not Provided” section of each extraction kit handbook to identify the accessories required for each procedure:
- QIASymphony DSP DNA Mini Kit (cat. no. 937236) for automated gDNA preparation (from FFPE samples)
- QIASymphony DSP Circulating DNA Kit (cat. no. 937556) for automated ccfDNA preparation (from plasma samples)
- QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual gDNA preparation (from FFPE samples)
- QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual ccfDNA preparation (from plasma samples)

Protocol: gDNA extraction from FFPE samples

The *therascreen* EGFR Plus RGQ PCR Kit has only been tested in combination with QIAGEN Deparaffinization Solution (cat. no. 19093 or 939018) for FFPE section deparaffinization with the following DNA extraction kits:

- QIASymphony DSP DNA Mini Kit (cat. no. 937236) for automated extraction
- QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual extraction

Important points before starting

Applicable to automated extraction and manual extraction protocols:

- Make sure the DNA extraction reagents have not expired and have been transported and stored under the correct conditions.
- Do not use expired or incorrectly stored components.
- Between one and four FFPE tissue sections, each with a thickness of 10 µm, or between two and eight sections with a thickness of up to 5 µm can be combined in one preparation.
- Use only the Deparaffinization Solution for FFPE deparaffinization, according to the procedure, "Pretreatment protocol for use with the QIASymphony DSP DNA Mini Kit" on page 33 or "Protocol: Pretreatment protocol for use with the QIAamp DSP DNA FFPE Tissue Kit" on page 36.

Note: Deparaffinization Solution is not supplied with extraction kits and should be ordered separately (see "Ordering Information", page 110).

- Use RNase A to minimize RNA content (included in procedure "Protocol: FFPE section deparaffinization with QIAGEN Deparaffinization Solution" on page 33).

Note: RNase A is not supplied with extraction kits and should be ordered separately (see "Ordering Information", page 110).

- Sample dilution may be necessary prior to qPCR testing (see "Protocol: gDNA quantification and normalization", page 41) or for storage.
- DNA isolated from FFPE samples is usually of lower molecular weight than DNA from fresh or frozen samples. The degree of fragmentation depends on the type and age of the sample and the conditions used for fixation.
- For DNA storage after extraction, see "Genomic DNA and circulating cell-free DNA samples", page 29.

Protocol: Automated gDNA extraction from FFPE samples using the QIASymphony SP

If using the QIASymphony DSP DNA Mini Kit (cat. no. 937236) for automated extraction, carry out the DNA extraction according to the instructions in the handbook, noting the following:

- Use only the Deparaffinization Solution for FFPE deparaffinization according to the procedure, “Pretreatment protocol for use with the QIASymphony DSP DNA Mini Kit”, page 33.
Note: Deparaffinization Solution is not supplied with the extraction kits and should be ordered separately (see “Ordering Information”, page 110).
- Select the Tissue_IC_200_V7_DSP protocol on the QIASymphony SP instrument (for more details, refer to the *QIASymphony SP Protocol Sheet Tissue_IC_200_V7_DSP* protocol)
- Use 50 µl elution volume.
- For any supplementary information concerning the QIASymphony SP instrument, refer to the user manual supplied with the instrument.

Protocol: Manual gDNA extraction from FFPE samples

If using the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) for manual extraction, carry out the DNA extraction according to the instructions in the handbook, noting the following:

- Use only the Deparaffinization Solution for FFPE deparaffinization according to the procedure “Protocol: Pretreatment protocol for use with the QIAamp DSP DNA FFPE Tissue Kit”, page 36.
Note: Deparaffinization Solution is not supplied with the extraction kits and should be ordered separately (see “Ordering Information”, page 110).
- Use 50 µl elution volume.

Protocol: FFPE section deparaffinization with QIAGEN Deparaffinization Solution

Pretreatment protocol for use with the QIASymphony DSP DNA Mini Kit

This pretreatment protocol is for use with the QIASymphony DSP DNA Mini Kit (for automated extraction) and is based on the *QIASymphony SP Protocol Sheet Tissue_LC_200_V7_DSP* protocol (Method 1: deparaffinization using the Deparaffinization Solution).

Important points before starting

- Equilibrate all buffers to room temperature (15–25°C) and equilibrate Deparaffinization Solution to 20–25°C.
- QIASymphony magnetic particles co-purify RNA and DNA if both are present in the sample. In order to minimize RNA content in the sample, add RNase A to the sample at the step indicated in the pretreatment protocol below.
- Deparaffinization Solution, RNase A and Buffer ATL are not supplied with the QIASymphony DSP DNA Mini Kit and must be ordered separately (see “Ordering Information”, page 110).

Things to do before starting

- Preheat a thermomixer or shaker-incubator to 56°C for use in step 7.
- Check ATL buffer for white precipitate. If necessary, dissolve precipitate according to the protocol described in *QIASymphony SP Protocol Sheet Tissue_LC_200_V7_DSP* protocol .

Procedure

Starting with FFPE blocks only

1. Using a scalpel, trim excess paraffin off the sample block. Cut between one and four sections of 10 µm thickness or between two and eight sections of 5 µm thickness.

Note: If the sample surface has been exposed to air, discard the first 2–3 sections.

2. Immediately place the section(s) in a 2 ml sample tube compatible with the sample tube carrier of the QIAAsymphony SP (not supplied; e.g., Sarstedt, cat. no. 72.693).
3. Continue with step 4 below (For all samples).

Starting with FFPE sections on slides only

1. Put 1 drop of Deparaffinization Solution on each slide using dedicated pipettes for sample preparation.
2. Scrape off the sample material with a single-use, sterile scalpel to collect the entire tissue. Place the aggregates in a 2 ml sample tube compatible with the sample tube carrier of the QIAAsymphony SP (not supplied; e.g., Sarstedt, cat. no. 72.693).
3. Continue with step 4 below (for all samples).

For all samples

4. Add 200 μ l Buffer ATL to the sections.
5. Add 20 μ l proteinase K.

Note: Use proteinase K from the enzyme rack of the QIAAsymphony DSP DNA Mini Kit.

6. Add 160 μ l or 320 μ l Deparaffinization Solution (see Table 2) and mix by vortexing.

Table 2. Volume of Deparaffinization Solution required

Section thickness	Number of sections	Volume of Deparaffinization Solution
5 μ m	1–4	160 μ l
	5–8	320 μ l
10 μ m	1–2	160 μ l
	3–4	320 μ l

7. Place the tube in a ThermoMixer or shaker-incubator and incubate at 56°C for 1 hour with shaking at 1000 rpm until the tissue is completely lysed.

Note: Lysis time varies depending on the tissue type processed. For most tissues, lysis is completed within 1 hour. If lysis is incomplete after 1 hour as indicated by the presence of insoluble material, lysis time can be prolonged or insoluble material can be pelleted by centrifugation. Overnight lysis is possible and does not affect the preparation.

8. Incubate at 90°C for 1 hour.

Note: The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA. If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block reaches 90°C.

9. To minimize RNA content in the sample, add 2 µl RNase A (100 mg/ml) to the lower phase and incubate for 2 minutes at room temperature before continuing with step 10. Allow the sample to cool to room temperature before adding RNase A.

10. Centrifuge at full speed for 1 minute at room temperature.

11. Carefully transfer tubes (containing both phases) to the sample carrier of the QIASymphony SP.

12. Proceed to extraction following the instructions in the *QIASymphony DSP DNA Mini Kit Handbook* (use 50 µl elution volume).

Protocol: Pretreatment protocol for use with the QIAamp DSP DNA FFPE Tissue Kit

This pretreatment protocol is for use with the QIAamp DSP DNA FFPE Tissue Kit (for manual extraction) and is based on “QIAGEN Supplementary Protocol: Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution”.

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Equilibrate all buffers to room temperature; equilibrate Deparaffinization Solution to 20–25°C.
- Deparaffinization Solution, RNase A and Buffer ATL are not supplied with the QIAamp DSP DNA FFPE Tissue Kit and must be ordered separately (see “Ordering Information”, page 110).

Things to do before starting

- Preheat a thermomixer or heated orbital incubator to 56°C for use in steps 6 and 10. If a thermomixer or heated orbital incubator is not available, a heating block or water bath can be used instead.
- If Buffer AL or Buffer ATL contain precipitates, dissolve precipitate according to the protocol described in QIAamp DSP DNA FFPE Tissue Kit.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.

Procedure

Starting with FFPE blocks only

1. Using a scalpel, trim excess paraffin off the sample block. Cut into sections 5–10 µm thick.

Note: If the sample surface has been exposed to air, discard the first 2–3 sections.

2. Immediately place the section(s) in a 1.5 ml or 2 ml microcentrifuge tube (not supplied).
3. Continue with step 4 below (for all samples).

Starting with FFPE sections on slides only

1. Put 1 drop of Deparaffinization Solution on each slide using dedicated pipettes for sample preparation.
2. Scrape off the sample material with a scalpel to collect the entire tissue. Put the aggregates in a 1.5 ml or 2 ml microcentrifuge tube (not supplied).
3. Continue with step 4 below (for all samples).

For all samples

4. Add 160 μ l or 320 μ l Deparaffinization Solution (Table 3) and vortex vigorously for 10 seconds.

Table 3. Volume of Deparaffinization Solution required

Section thickness	Number of sections	Volume of Deparaffinization Solution
5 μ m	1–4	160 μ l
	5–8	320 μ l
10 μ m	1–2	160 μ l
	3–4	320 μ l

5. Centrifuge briefly to collect the sample in the bottom of the tube.
6. Incubate at 56°C for 3 minutes and allow to cool at room temperature (15–25°C).
7. Add 180 μ l Buffer ATL, and mix by vortexing.
8. Centrifuge for 1 minute at 11,000 \times g (10,000 rpm). Two phases appear (blue and clear).

-
9. Add 20 μ l proteinase K to the lower, clear phase. Mix gently by pipetting up and down.
 10. Incubate at 56°C for 1 hour (or until the sample has completely lysed).
 11. Incubate at 90°C for 1 hour.

The incubation at 90°C in Buffer ATL partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented DNA.

Note: If using only one heating block, leave the sample at room temperature (15–25°C) after the 56°C incubation in step 10, until the heating block has reached 90°C for step 10.

12. Briefly centrifuge the 1.5 ml tube to remove drops from inside the lid.
13. Transfer the lower, clear phase into a new 2 ml microcentrifuge tube.
14. Add 2 μ l RNase A (100 mg/ml) and incubate for 2 minutes at room temperature.
15. Continue with step 12 (addition of Buffer AL) of the *QIAamp DSP DNA FFPE Tissue Kit Handbook* (use 50 μ l elution volume).

Protocol: ccfDNA extraction from plasma samples

The *therascreen* EGFR Plus RGQ PCR Kit has been tested in combination with the following DNA extraction kits:

- QIASymphony DSP Circulating DNA Kit (cat. no. 937556) for automated ccfDNA extraction (from plasma samples)
- QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual ccfDNA extraction (from plasma samples)

Important points before starting

Applicable to automated extraction and manual extraction protocols:

- Make sure the DNA extraction reagents have not expired and have been transported and stored under the correct conditions.
- Do not use expired or incorrectly stored components.
- Starting material for ccfDNA purification should be plasma prepared from 2K-EDTA whole blood samples. Samples can be either fresh or frozen (provided that they have not been frozen and thawed more than once).
- The concentration of circulating cell-free nucleic acids in biological fluids such as plasma is usually low and varies considerably between individuals. Therefore, ccfDNA extracted from plasma samples will not be quantified nor normalized (no dilution), and is used directly in the qPCR reaction.
- For DNA storage after extraction, refer to the section “Genomic DNA and circulating cell-free DNA samples”, page 29.

Protocol: Automated ccfDNA extraction from plasma samples using the QIAasymphony SP

If using the QIAasymphony DSP Circulating DNA Kit (cat. no. 937556) for automated extraction, carry out the DNA extraction according to the instructions in the handbook, noting the following:

- Select the circDNA_2000_DSP_V1 protocol on the QIAasymphony SP instrument (for protocol details, refer to *QIAasymphony SP Protocol Sheet circDNA_2000_DSP_V1*)
-  The recommended sample volume for the circDNA_2000_DSP is 2 ml. However, we recommend starting with 2.4 ml to prevent any extraction failure during initial pipetting, as specified in the “Troubleshooting Guide” of the *QIAasymphony DSP Circulating DNA Kit Handbook*. If insufficient sample is available, add sterile PBS (not supplied) to the sample up to the required sample volume before loading the sample.
- Use a 60 µl elution volume.
- For any supplementary information concerning the QIAasymphony SP instrument, refer to the user manual supplied with the instrument.

Protocol: Manual ccfDNA extraction from plasma samples

If using the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) for manual purification, carry out the DNA extraction according to the instructions in the handbook noting the following:

- Purification of circulating nucleic acids is performed from 2 ml of plasma.
- A vacuum manifold (e.g., the QIAvac 24 Plus with the QIAvac Connecting System) and a vacuum pump capable of producing a vacuum of –900 to –800 mbar (e.g., QIAGEN Vacuum Pump) are required for the protocol.
- Use a 60 µl elution volume.

Protocol: gDNA quantification and normalization

Things to do before starting

If using automated extraction procedures, check the “Validity of result” column for each sample on the QIA Symphony SP result file once the run is finished:

- Valid status: Proceed to gDNA quantification.
- Unclear status: Can be processed depending on the origin of the flag (for details regarding possible origins of “unclear” flagging, refer to the QIA Symphony SP/AS User Manual).
- Invalid status: Sample is rejected. Repeat the extraction step.

Procedure

gDNA extracted from FFPE samples should be quantified.

If measured concentration is less than 4 ng/μl, the sample must be re-extracted with more sections (to a maximum of eight sections of 5 μm or four sections of 10 μm).

If measured concentration is above 6 ng/μl, the sample must be diluted to 5 ng/μl using the Water for sample dilution provided in the *therascreen* EGFR Plus RGQ PCR Kit, according to the formula:

$$C_i \times V_i = C_f \times V_f$$

Where

C_i: Initial concentration of the extracted gDNA

C_f: Final concentration to be targeted = 5 ng/μl

V_f: Final volume needed to perform a *therascreen* EGFR Plus RGQ PCR run (i.e., 20 μl + extra-volume for pipetting error)

V_i : Initial volume of the extracted gDNA to be pipetted and diluted with the Water for sample dilution provided in the *therascreen* EGFR Plus RGQ PCR Kit (Water volume to add = $V_f - V_i$)

Each PCR reaction is optimized for 25 ng gDNA diluted in a final sample volume of 5 μ l. As each sample is tested with the four *EGFR* reaction mixes, a total of 100 ng per tested sample is needed.

Note: Make sure the correct elution buffer is used to calibrate the quantification instrument.

ccfDNA extracted from plasma samples should not be quantified. Each PCR reaction is optimized for 5 μ l of pure extracted ccfDNA. As each sample is tested with the four *EGFR* reaction mixes, a total of 20 μ l per tested sample is needed.

Protocol: *EGFR* mutation assessment by qPCR on Rotor-Gene Q MDx 5Plex HRM instrument

Important points before starting

- Make sure the operator is trained in using the instruments for qPCR. If required, instrument training can be provided upon installation (see “Ordering Information”, page 110).
- Read “Precautions”, page 23, and become familiar with all components of the kit prior to use.
- The *therascreen* EGFR Plus RGQ PCR Kit must be run on a Rotor-Gene Q MDx 5plex HRM instrument* using the Rotor-Gene AssayManager version 2.1 (or later) combined with Gamma plug-in version 1.0.0 (or later) associated to the FFPE or plasma dedicated Assay Profile.
- Take time to familiarize yourself with the Rotor-Gene Q MDx 5plex HRM instrument, with the Rotor-Gene AssayManager software and with the Gamma plug-in before starting the protocol. See the user manuals for the instrument, Rotor-Gene AssayManager and the Gamma plug-in for details.
- Rotor-Gene AssayManager version 2.1 enables automated interpretation of the PCR results. The cycling parameters are locked for the run.
- If you are using the Rotor-Gene AssayManager version 2.1 software, the Gamma Plug-in and the assay profile for the first time, please refer to the section “Appendix A: Installation of the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in, and Importing the Assay Profile” on page 103 for installation instructions. If the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in and the assay profile are already installed and imported in your computer, continue with the instructions below.

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

- If you are using automated extraction procedures, check the “Validity of result” column for each sample on the QIASymphony SP result file once the run is finished, see “Protocol: gDNA quantification and normalization”, page 41.
- If you are using gDNA extracted from FFPE, the sample should be quantified and diluted to 5 ng/μl, see “Protocol: gDNA quantification and normalization”, page 41.
- If you are using ccfDNA extracted from plasma, samples should be used undiluted.

Setting up the qPCR

Using the *therascreen* EGFR Plus RGQ PCR Kit, it is recommended to test six DNA samples in the same experiment to optimize use of the controls and reaction mixes. However, up to 16 samples can be tested in the same experiment.

Things to do before starting

- Cool the Loading Block (72 x 0.1 ml tubes) in a refrigerator (2–8°C).
- Before each use, thaw all necessary components.
Note: Do not exceed 1 hour at room temperature for the thawing step to avoid any material degradation. If more time is required, store components at 2–8°C for up to 8 hours.
- Clean the bench area dedicated for the PCR mix preparation to reduce the risk of template or nuclease contamination.
- Vortex the tubes containing controls, Primers and Probes mixes and PCR master mix (3–5 seconds) and then centrifuge briefly before use.

Procedure

1. Prepare the four PCR Reaction Mixes in 1.5 ml or 2 ml tubes (not provided), i.e., mix each Primers and Probes mix (T790M & L861Q Mix, Insertions & G719X Mix, L858R & C797S Mix or Deletions & S768I Mix) with PCR Master Mix, according to the number of samples to be processed.

The volume required for each kit component to make the Reaction Mixes is shown in Table 4. The final PCR reaction volume is 25 μl after addition of 5 μl sample DNA or

run control template. Extra volume is included to compensate for pipetting variation and to allow preparation of enough reaction mix for planned number of test samples and controls, e.g., six samples plus two controls.

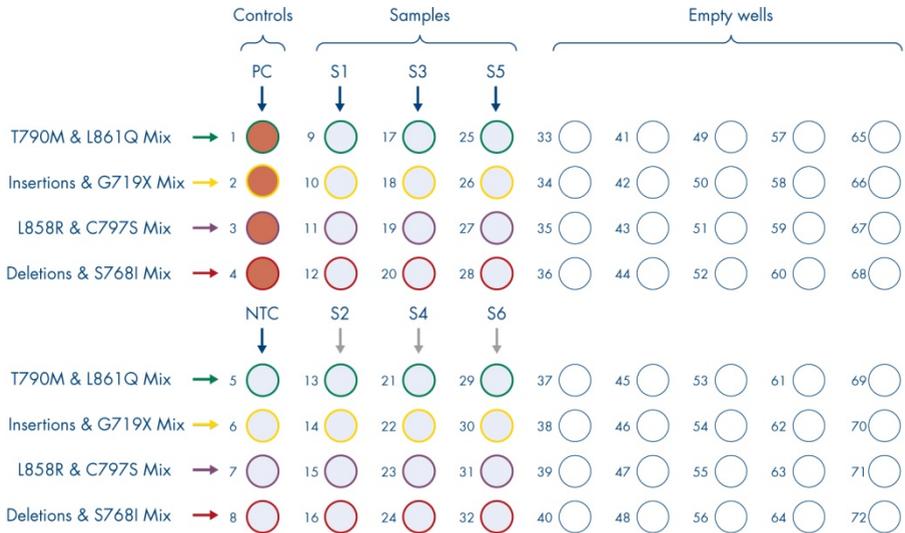
Table 4. Preparation of Reaction Mixes

Component	1 reaction (µl)	8 + 1 reactions (µl)*
EGFR Primers and Probes Mix	7.5	67.5
PCR Master Mix	12.5	112.5
Total volume of Reaction Mix	20	180
Reaction Mix distribution	20 µl per tube	
Test sample distribution	5 µl per tube	
Total volume per reaction	25 µl	

* An extra reaction volume is included to compensate for pipetting error: one extra well for up to 10 wells and two extra wells for up to 20 wells.

2. Return all the *therascreen* EGFR Plus RGQ PCR Kit components to the freezer to avoid any material degradation.
3. Vortex Reaction Mixes for 3–5 seconds and centrifuge briefly.
4. Place the PCR strip tubes on a cooled Loading Block (72 x 0.1 ml tubes) and dispense 20 µl EGFR Reaction Mixes per strip tube following the loading block setup shown in Figure 6.

Note: It is recommended to dispense the 20 µl of the reaction mix by reverse pipetting.



Note: Both the FFPE and plasma DNA samples can be run in the same experiment. This requires running both the FFPE and the Plasma assay profiles in the same experiment and a specific plate layout. Refer to Appendix B: Running the FFPE and Plasma Assay Profiles in the Same Experiment (page 107) for details.

5. Add 5 µl of Water for NTC into the designated NTC tubes (Figure 6) to obtain a total volume of 25 µl. Mix gently by pipetting up and down. Close all tubes containing NTC.
6. Vortex and briefly centrifuge DNA samples and *EGFR* Positive Control (PC). Then, add 5 µl sample or PC template into the corresponding tubes (Figure 6) to obtain a total volume of 25 µl. Mix gently by pipetting up and down.
7. Close all tubes and check that no bubbles are present at the bottom of the tubes.

Note: Change tips between each template addition to avoid contamination.

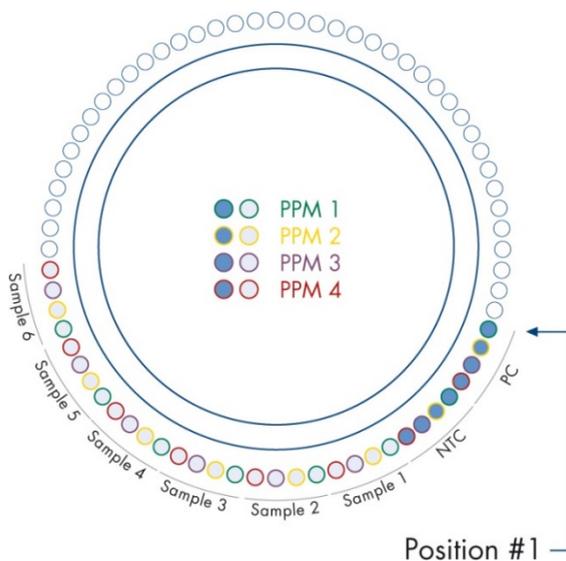
Protocol: Preparing the Rotor-Gene Q MDx 5plex HRM instrument

- Place a 72-well rotor on the Rotor-Gene Q MDx 5plex HRM instrument rotor holder.
- Fill the rotor with strip tubes according to the assigned positions, starting at position 1, as shown in Figure 7.

Note: Make sure the first tube is inserted into position 1 and the strip tubes are placed in the correct orientation and positions as shown.

- All unused positions should be filled with empty capped strip tubes.

Note: We recommend keeping the four positive controls in positions 1 to 4 and the four no template controls in positions 5 to 8 as the automated analysis set in the assay profiles are based on this organization. If a different layout is used, aberrant or invalid results will be obtained.



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11. Attach the locking ring.
 12. Load the Rotor-Gene Q MDx 5plex HRM instrument with the rotor and locking ring.
Close the instrument lid.

Creating a worklist and starting the qPCR run

Note: The work list can be created and saved before preparing the samples or when the experiment is set up on the instrument, as described in this handbook.

13. Switch on the Rotor-Gene Q MDx 5plex HRM instrument.
14. Open the **Rotor-Gene AssayManager v2.1** software.
15. Log in as user with the **Operator** role in closed mode. Click **OK**. The following window appears.

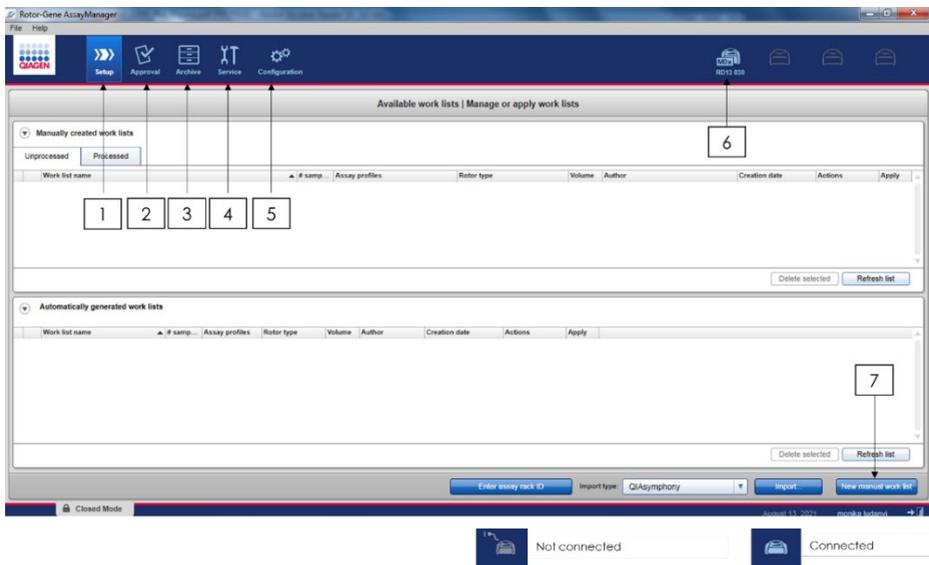


Figure 8. Rotor-Gene AssayManager v2.1.1 1 = setup tab. This tab allows managing or applying work lists 2 = Approval tab. This tab enables you to find previous experiments. 3 = Archive tab. This tab allows you to find previously approved experiments. 4 = Service tab. In this tab, an audit trail of each file generated by the software is reported. 5 = Configuration tab. This tab allows configuration of all software parameters. 6 = Rotor-Gene Q (RGQ) instrument icon; informs user whether a given cycler is connected. Up to four RGQ instruments can be connected to the same computer. 7 = New manual work list.

16. Check that the RGQ is correctly detected by software before launching the run. For more information, see “Cycler Environment” in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.
17. Click **New manual work list** in the work list manager (“Setup” environment) (Figure 8).
18. Select the relevant EGFR assay profile from the list of available assay profiles:
 - For testing of gDNA samples from FFPE: therascreen_EGFR_Plus_FFPE
 - For testing of ccfDNA samples from plasma: therascreen_EGFR_Plus_Plasma

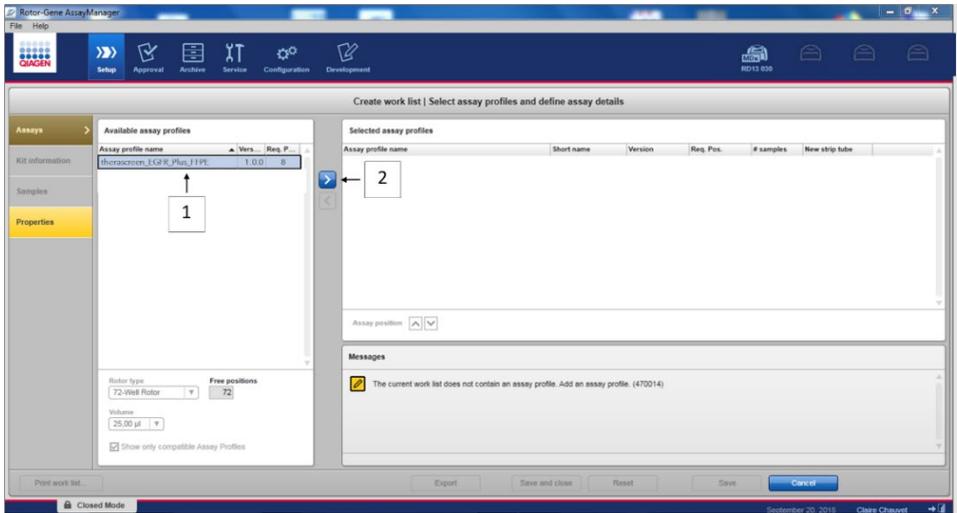


Figure 9. Selecting an assay profile. 1 = Available assay profiles; 2 = Assay profile transfer to worklist

Note: It is possible to run both the FFPE and the Plasma assay profiles in the same experiment. Refer to Appendix B: Running the FFPE and Plasma Assay Profiles in the Same Experiment, page 107, for details.

19. Click **Move** to transfer the selected assay profile to the **Selected assay profiles** list.
20. Enter the number of samples in the corresponding field.



Figure 10. Creation of the work list: definition of assay details. 3 = number of sample

Note: The number of samples does not correspond to the number of tubes and does not include controls.

21. Select the “Kit Information” tab. Enter the following EGFR kit information, which is printed on the label of the *therascreen* EGFR Plus RGQ PCR kit box:

- Material number: 1114551
- Valid expiry date
- Lot number

Figure 11. Creation of the work list: Edit kit information. 4 = kit bar code. This tab indicates the bar code of the kit (if the bar code is entered, the other fields are filled automatically). 5 = Material number. 6 = Kit expiry date. 7 = Lot number. These information are available on the kit box.

Note: All fields must be filled in and become blue when valid information is entered.

22. Select the “Samples” tab. A list with the sample details is shown. This list represents the expected layout of the rotor.
23. Enter the sample identification into this list, as well as any optional sample information as a comment for each sample.

Note: The “**is editable**” box (Figure 13) defines whether the work list is still editable or not. So if the work list is applicable and shall not be changed afterwards, this box must be cleared.

Note: The work list can be applied directly or can be saved and run later.

25. Check the **worklist is complete (can be applied)** checkbox.

26. Save the work list.

Optional: The work list can be printed and this may help with the preparation and setup of the qPCR. To print the work list, click **Print work list**. The sample details are included as part of this work list.

27. Select the corresponding work list from the work list manager and click **Apply**.

Alternatively, if the work list is still open, click **Apply**.

28. Enter the experiment name in the **Experiment name** field.

29. In the **Cycler selection** list, select the cycler to use.

Note: A Rotor-Gene Q MDx 5plex HRM instrument must be used*.

30. Ensure that the locking ring is correctly attached and check the **Ring attached** box.

31. Click **Start run**. The qPCR run starts (Figure 14).

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

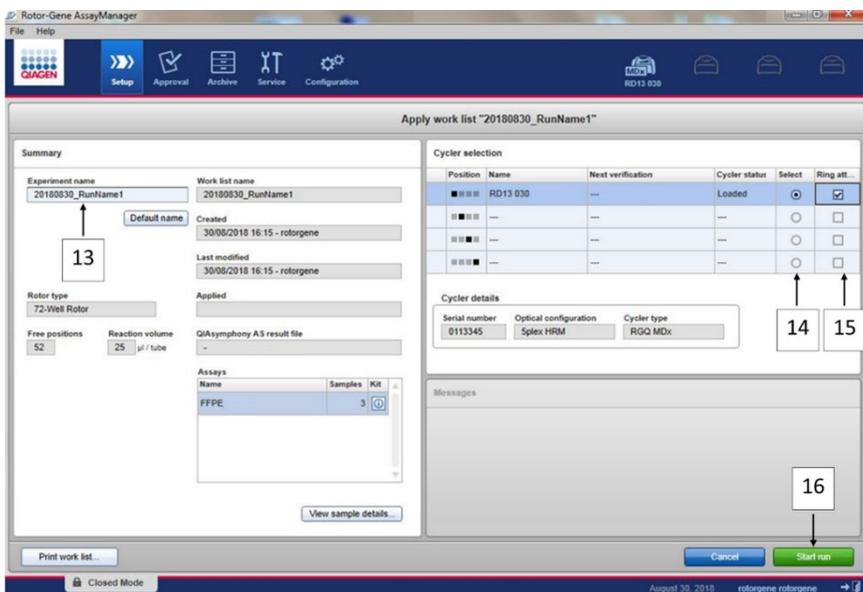


Figure 14. Run launch. 13 = Enter experiment name; 14 = cyclers selection; 15 = confirm locking ring is attached; 16 = click start run to launch the run.

Releasing and reporting qPCR results

The general functionality of the Approval environment is described in the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

After a run has finished and the cycler has been released, the experiment will be stored in the internal database. The analysis of the acquired data is performed automatically depending on the plug-in corresponding to the assay profile and the rules and parameter values defined by the assay profile.

32. After the run is complete, click **Finish run** (Figure 15).

Note: Until this step is completed, the experiment is not saved in the internal database.

33. Release and approve the run.

- For users logged in with the Approver role, click **Release and go to approval**.
- For users logged in with the Operator role, click **Release**.

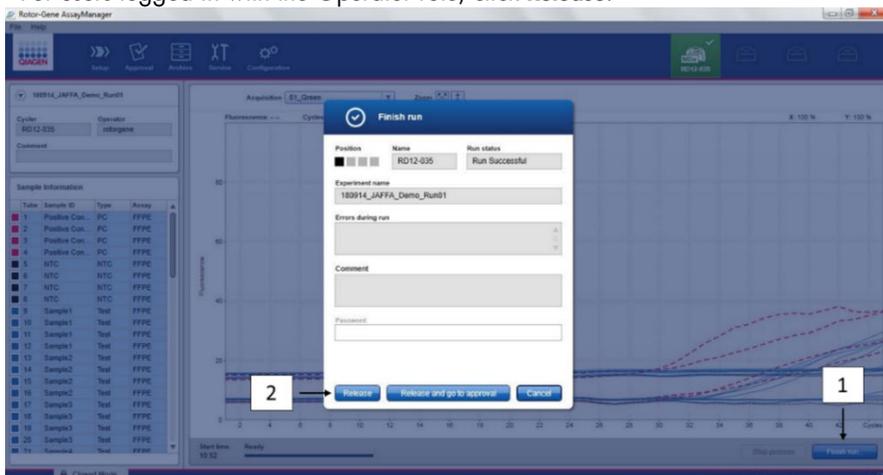


Figure 15. Finalization of the run. Finish run (1) and Release run (2)

34. Release results.

- If you clicked **Release and go to approval**, the results for the experiment are displayed in the Approval environment.
- If you clicked **Release** with a user role, a user with an Approver role needs to log in and select the Approval environment.

35. Filter the assay to be approved by selecting the filter options and clicking **Apply**. Select the desired assay within the list of filtered assays using the check box and click **Start Approval**.

36. Use radio buttons to (Figure 16) to accept or reject samples.

Note: Samples can be rejected in case of operator handling error or unusual curves (artefact).

37. Review results and click **Release/Report data**.

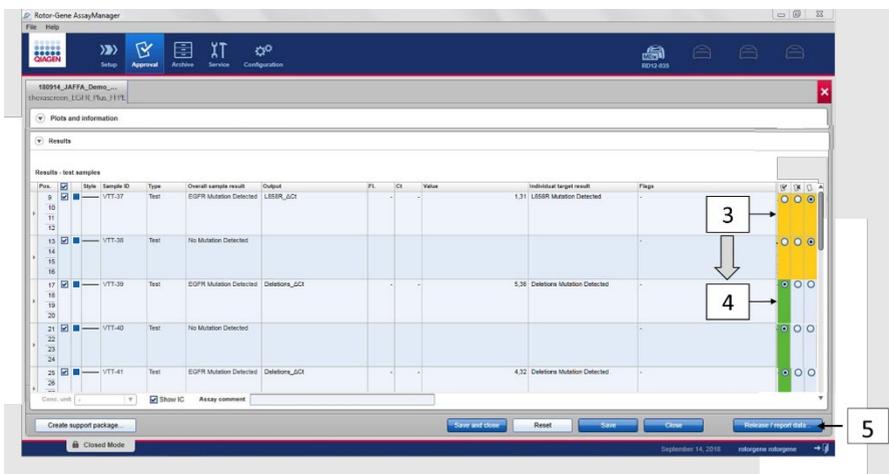


Figure 16. Review and release data. Review and accept (ü) or reject (û) results for each sample: box color changes from yellow to e.g. green if data is approved (3, 4). Then, click “Release / report data” (5).

38. Enter password if required, and click OK. The report is generated in Adobe Portable Document Format (.pdf) and automatically stored in the predefined folder. The default folder path is **C:\Documents and settings\Public\Documents\QIAGEN\Rotor-Gene AssayManager\Export\Reports**.

Note: You can change the path and folder in the Configuration environment.

Note: At the same time, a LIMS file is automatically created and stored in the predefined folder. The default folder path is **C:\Documents and settings\Public\Documents\QIAGEN\Rotor-Gene AssayManager\Export\LIMS**

39. Close the pdf file and return to the Rotor-Gene AssayManager. Click **OK** for each prompt.

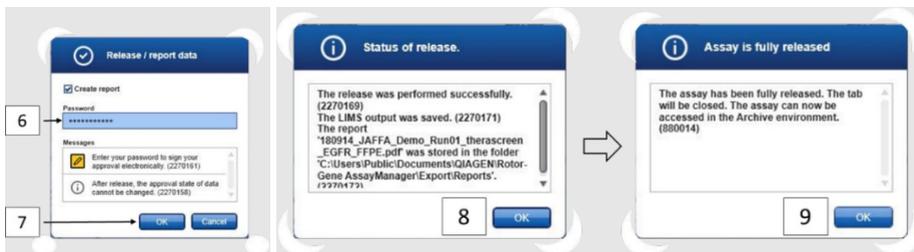


Figure 17. Release/Report data. Enter password (6) then click OK (7). A PDF report is generated and opens; close PDF report: a LIMS file is automatically generated, and a statement of release appears, click OK (8). Assay is now fully released: click OK to go to the Archive environment (9).

40. Go to the “Archive” tab to export the .rex file, corresponding to the raw data. Find your experiment using the filter options and click “show assays” (Figure 18)

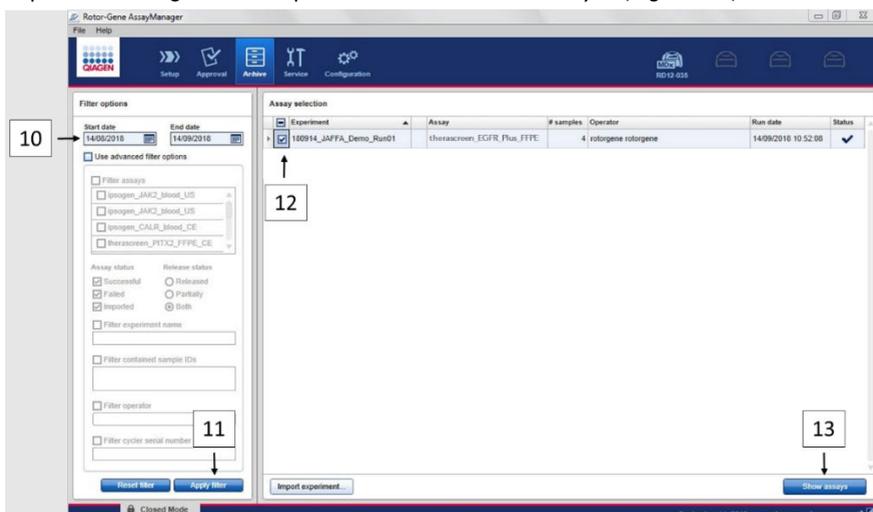


Figure 18. Selecting your experiment in the Archive environment. For instance, filter by date (10) and apply filter (11). Select the experiment (12) then click on “Show assays” (13).

41. Click **Export .rex file** and click **OK** to save.

Note: You can select a location to save the .rex file (the default path is **C:\Documents and settings\Public\Documents\QIAGEN\Rotor-Gene**)

AssayManager\Export\ExperimentsforClosedMode). This path and folder can also be changed in the tab “specify the .rex file export destination”.

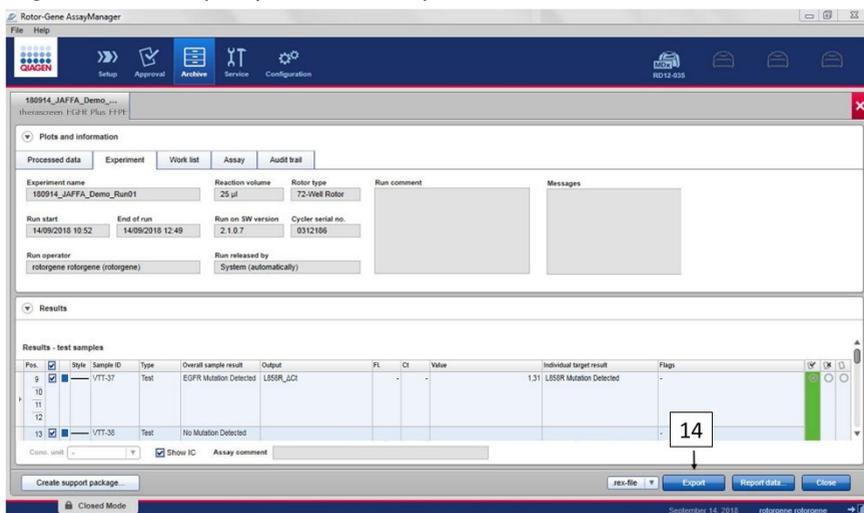


Figure 19. Export .rex file by clicking the “Export” button (14).

Note: A support package from the run is required for assistance with troubleshooting by QIAGEN Technical Support. Support packages can be generated from the Approval or Archive environments. For more information, see “Creating a support package” in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

In addition to the support package, the audit trail from ± 1 day of the time of an incident might be helpful. The audit trail can be retrieved in the Service environment. For more information, see the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

42. Unload the Rotor-Gene Q MDx 5Plex HRM instrument and discard the strip tubes according to your local safety regulations.

Interpretation of Results [if applicable]

Analysis of the *therascreen* EGFR Plus RGQ PCR Kit results for each control and sample is performed automatically by the Rotor-Gene AssayManager v2.1 associated with the Gamma Plug-in v1.0 and the EGFR assay profiles.

The EGFR assay profiles analyze amplification curves, and may invalidate non-conforming curves, depending on their shape and noise amplitude. If this is the case, a flag will be associated with the invalidated curve (see Table 6, page 63).

Controls

The Rotor-Gene AssayManager v2.1 analyzes the run controls:

- NTC is checked for the absence of specific amplification.
- Validity of the Positive Control is based on compliance of CT values with pre-defined specifications.
- If any of these run controls does not conform, the flag "ASSAY_INVALID" will be triggered. If this flag is triggered, the run is considered invalid and the experiment needs to be performed again (decision flow chart for retests is presented in Figure 20).
- **Note:** The report generated at the end of the run shows the results obtained on run controls, with invalidating flags (see Table 6, page 63) in front of invalid data.

If all of the controls in the run conform, then Rotor-Gene AssayManager v2.1 analyzes the test samples. DNA samples from both FFPE and plasma are analyzed following the same process but with specific criteria recorded in the respective assay profiles.

Samples

Exon 2 Internal control

Validity of the Exon 2 Internal Control is based on compliance of Ct values with pre-defined specifications. The internal control must be valid for the sample results to be interpreted. A valid internal control indicates sufficient DNA input and quality as well as the absence of interfering substances. In case of invalidity, refer to the decision flow chart presented in Figure 20.

EGFR mutation detection

The presence or absence of EGFR mutations in each test sample is assessed, based on the delta Ct between the mutant amplification and the internal control amplification (targets T790M_ΔCt, L861Q_ΔCt, etc) for FFPE samples, and based on the mutant amplification for plasma samples (CT) .

EGFR mutation semi-quantification

A semi-quantitative estimation of the mutation concentration in ccfDNA from plasma is given for the concerned targets (listed in Summary and explanation) in the form of an interval lower and upper limits. The number of mutant copies per milliliter of plasma is estimated, i.e. the interval lower and upper limits are given by targets T790M_CN_LL, L861Q_CN_LL, etc.

Results for each target are displayed in the **Result** column of the report.

The conclusion of the analysis for each sample is displayed in the **Overall Sample Result** column of the report (Table 5).

Table 5. Overall sample results and actions

Overall sample result	Sample type	Description	Action
Valid	Control (Positive Control, NTC)	Control is Valid	N/A
Invalid*	Control (Positive Control, NTC)	Control is Invalid (CT value outside predefined acceptance range)	Repeat the entire run (i.e. all four EGFR reaction mixes, all samples)
EGFR Mutation Detected**	Test Sample	At least one EGFR mutation was detected†	N/A
No Mutation Detected**	Test Sample	No EGFR mutation was detected	N/A
Invalid (associated with flag ASSAY_IC_BELOW_ACCEPTED_RANGE) [§]	Test Sample	The detected CT value is below the acceptance range for the internal control. DNA input may be too high.	Dilute sample prior to retest. Retest the concerned sample(s) with all four EGFR reaction mixes in a new qPCR run.
Invalid (associated with flag ASSAY_IC_ABOVE_ACCEPTED_RANGE) [§]	Test Sample	The detected CT value is above the acceptance range for the internal control. Sample may not contain enough amplifiable DNA or could contain an inhibitor.	Retest the concerned sample(s) with all four EGFR reaction mixes in a new qPCR run. If sample was already retested once before, repeat extraction step and retest in a new qPCR run. If presence of inhibitor is suspected, try diluting sample prior to retest (for plasma only). For additional information, refer to Table 6 on page 63.

Table continued on next page

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Table 5. Overall sample results and actions (cont'd)

Overall sample result	Sample type	Description	Action
Invalid (associated with flag ASSAY_IC_NO_CT_VALUE) ^{&}	Test Sample	No signal detected for the internal control.	Retest the concerned sample(s) with all four EGFR reaction mixes in a new qPCR run.
Invalid (other)	Test Sample	Invalidity due to curve anomaly or other cause (refer to Table 6, page 63)	If sample was already retested once before, repeat extraction step and retest in a new qPCR run. If presence of inhibitor is suspected, try diluting sample prior to retest (for plasma only). For additional information, refer to Table 6 on page 63

* When controls are invalid, the invalid CT values are displayed between square brackets for information.

** For mutations in scope of the *therascreen* EGFR Plus RGQ PCR Kit (listed in Table 1)

† For identification of detected EGFR mutation(s), refer to Δ Ct targets (e.g. T790M_ΔCt), column target result (e.g. T790M Detected). For semi-quantification results (number of copies per milliliter of plasma for ccf DNA), refer to targets X_CN_LL and X_CN_UL (where X = mutation name), column value, to obtain the lower and upper limits of the semi-quantification interval.

Note: & ASSAY stands for T790M_L861Q / INSERTIONS_G719X / L858R_C797S / DELETIONS_S768I

Flags

Invalid results are associated with flags displayed in the **Flag** column of the Rotor-Gene AssayManager report.

Invalidating sample flags that may be assigned to a sample or target during the analysis by Rotor-Gene AssayManager v2.1, are defined in Table 6. For universal flags included in the Gamma Plug-in, refer to the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

Table 6. Definition of flags

Flag	Description
ANALYSIS_FAILED	Assay is set to invalid because the analysis has failed. Contact QIAGEN Technical Services
ASSAY_INVALID	Sample is invalid because at least one external control is invalid.
CONSECUTIVE_FAULT	A target that was used for calculation of this target is invalid.
CURVE_SHAPE_ANOMALY	The raw data amplification curve shows a shape that deviates from the established behavior for this assay. There is a high likelihood of incorrect results or a result misinterpretation.
FLAT_BUMP	The amplification curve shows a shape like a flat bump deviating from the established behavior for this assay. There is a high likelihood of incorrect results or result misinterpretation (e.g., wrong C _T value determination).
LOW_FLUORESCENCE_CHANGE	The percentage fluorescence change for this sample relative to the sample tube with the largest fluorescence change is lower than a defined limit.
MULTIPLE_THRESHOLD_CROSSING	The amplification curve crosses the threshold more than once. An unambiguous C _T cannot be determined.
TARGET_NTC_UNEXPECTED_CT_VALUE*	An invalid signal was detected in the no template control
OTHER_TARGET_INVALID	Another target for the same sample is invalid.
ASSAY_IC_PC_BELOW_ACCEPTED_RANGE& ASSAY_IC_PC_ABOVE_ACCEPTED_RANGE&	The detected C _T value is outside the acceptance range for the internal control in the Positive Control tube.
ASSAY_IC_PC_NO_CT_VALUE&	No signal detected for the internal control in the Positive Control tube.
TARGET_PC_BELOW_ACCEPTED_RANGE** TARGET_PC_ABOVE_ACCEPTED_RANGE**	The detected C _T value is outside the acceptance range for a mutation assay in the Positive Control tube.

Table continued on next page

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Table 6. Definition of flags (cont'd)

Flag	Description
TARGET_PC_NO_CT_VALUE**	No signal detected for the target channel in the Positive Control tube.
RUN_FAILED	Invalid Assay is set to invalid due to a problem with the cycler or the cycler connection.
RUN_STOPPED	Invalid Assay is set to invalid because the run has been stopped manually.
ASSAY_IC_BELOW_ACCEPTED_RANGE&	The detected C_T value is below the acceptance range for the internal control in a test sample. DNA input may be too high: dilute sample prior to retest.
ASSAY_IC_NO_CT_VALUE&	No signal detected for the internal control in a test sample.
ASSAY_IC_ABOVE_ACCEPTED_RANGE	The detected C_T value is above the acceptance range for the internal control in a test sample. Sample may not contain enough amplifiable DNA or could contain an inhibitor.
SATURATION	The raw data fluorescence is saturating strongly before the inflection point of the amplification curve.
SPIKE	A spike in the raw data fluorescence is detected in the amplification curve but outside the region where the C_T is determined.
SPIKE_CLOSE_TO_CT	A spike is detected in the amplification curve close to the C_T .
NO_BASELINE	No initial baseline has been found. The subsequent analysis cannot be performed
STEEP_BASELINE	A steeply rising baseline for the raw data fluorescence is detected in the amplification curve.
STRONG_BASELINE_DIP	A strong drop in the baseline for the raw data fluorescence is detected in the amplification curve.

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Table 6. Definition of flags (cont'd)

Flag	Description
STRONG_NOISE	Strong noise outside the growth phase of the amplification curve detected.
STRONG_NOISE_IN_GROWTH_PHASE	Strong noise is detected in the growth (exponential) phase of the amplification curve.
TARGET_ΔCT_UNEXPECTED_EARLY_CT**	The detected ΔCT is lower than expected for a test sample target mutant reaction mix
TARGET_CT_UNEXPECTED_EARLY_CT**	The detected CT is lower than expected for a test sample target mutant reaction mix
WAVY_BASE_FLUORESCENCE	Wavy baseline for the raw data fluorescence detected in the amplification curve.

* TARGET stands for T790M, L861Q, T790M_L861Q_IC, INSERTIONS, G719X, INSERTIONS_G719X_IC, L858R, C797S, L858R_C797S_IC, DELETIONS, S768I, DELETIONS_S768I_IC

** TARGET stands for T790M, L861Q, INSERTIONS, G719X, L858R, C797S, DELETIONS, S768I

& ASSAY stands for T790M_L861Q / INSERTIONS_G719X / L858R_C797S / DELETIONS_S768I

Retests

In case of invalid results, refer to the “Troubleshooting Guide”, page 95, to investigate the cause of failure and potentially identify any error that needs to be corrected.

The procedure for retests is summarized in Figure 20.

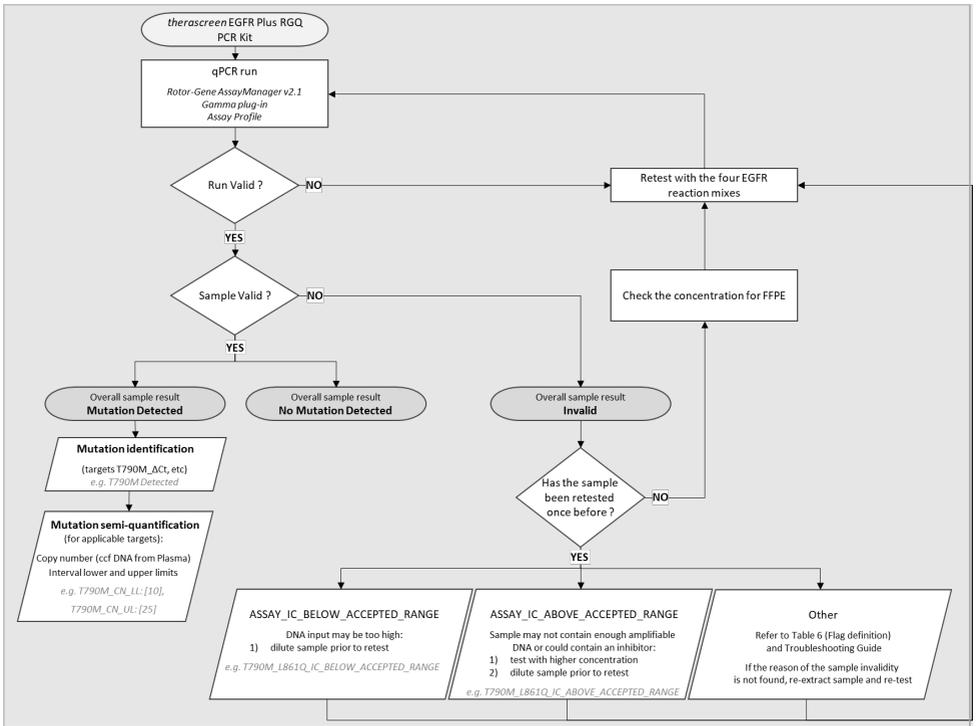


Figure 20. theascreen EGFR Plus RGQ PCR Kit decision flowchart.

If one or several run controls are invalid, the run should be repeated with the 4 EGFR Reaction Mixes. For example, if the positive control does not meet the validity criteria for the T790M & L861Q Mix, but is valid for all other EGFR Reaction Mixes, the four Mixes should be retested with all samples.

If one or several samples are invalid, the concerned samples should be retested with the 4 EGFR Reaction Mixes. Depending on the flag which is displayed by the RGAM, dilute your sample prior retest or retest it with higher concentration.

If the reason of the sample invalidity is not found:

-
- Check that your samples have been handled and stored as described in section “Sample Storage and Handling”.
 - Re-extract your FFPE sample with more sections prior to retest.
 - Re-extract your FFPE sample by selecting a bigger tumor zone prior to retest.
 - Please note that all performances have been established using DNA extracted from FFPE at 5ng/μl and/or 5μl of pure ccfDNA extracted from plasma.

For other explanations regarding the sample invalidity, please refer to the “Troubleshooting Guide” page 95.

Limitations

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

The product is to be used by laboratory professionals trained in molecular biology procedures, in vitro diagnostics procedures, and trained in the use of the QIASymphony SP system, Rotor-Gene Q MDx 5Plex HRM instrument, the Rotor-Gene AssayManager, and the Gamma plug-in.

The product is intended for use only on a Rotor-Gene Q MDx real-time PCR cycler, 5plex HRM series combined with the Rotor-Gene AssayManager software and the Gamma plug-in using the *therascreen* EGFR Plus dedicated assay profiles.

We recommend the use of the Deparaffinization Solution (including RNase A treatment), QIAamp DSP DNA FFPE Tissue Kit, QIAamp DSP Circulating Nucleic Acid Kit, QIASymphony DSP DNA Mini Kit, and QIASymphony DSP Circulating DNA Kit.

Strict compliance with the *therascreen* EGFR Plus RGQ PCR Kit Instructions for Use is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended and will result in a loss of performance. All reagents supplied in the *therascreen* EGFR Plus RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same kit. Using reagents from different kit lots within the same run may affect performance.

It is important that the amount of gDNA from the FFPE sample is assessed prior to performing sample analysis using the *therascreen* EGFR Plus RGQ PCR Kit. The extraction procedure should be repeated if the gDNA quantity is not sufficient for mutation analysis. gDNA should be diluted if the concentration is too high for mutation analysis.

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

The *therascreen* EGFR Plus RGQ PCR Kit is validated only for plasma collected in 2K EDTA and FFPE from NSCLC patients.

Any off-label use of this product and/or modification of the components will void QIAGEN's liability.

Performance Characteristics

Limit of blank

The limit of blank (LOB) was determined using 77 NSCLC EGFR wild-type FFPE samples and 75 healthy donor Plasma samples (at least 60 measurements per reagent lot, 3 *therascreen* EGFR Plus RGQ PCR Kit lots used). The LOB was determined for each given assay, as the lowest LOB value obtained. The LOB results are summarized in Table 7.

Table 7. Summary of the limit of blank results for the *therascreen* EGFR Plus RGQ PCR Kit

EGFR target	FFPE LoB (Δ Ct)	Plasma LoB (Δ Ct)
T790M	11.49	40.23
L861Q	15.31	35.54
Insertions	11.32	38.42
G719X	14.47	45.00
L858R	10.52	37.54
C797S	15.06	45.00
Deletions	14.15	45.00
S768I	14.64	45.00

The false positive rate is below 1% for all EGFR targets except for L858R in FFPE (1.2%) and for Insertions in Plasma samples (1.08%)

Limit of Detection

The limit of detection (LOD) of each of the 42 EGFR mutations was determined on EGFR low positive FFPE and Plasma samples (3 *therascreen* EGFR Plus RGQ PCR Kit lots used). The LOD results are summarized in Table 8.

Table 8. Summary of the limit of detection results for the *therascreen* EGFR Plus RGQ PCR Kit

Exon	Mutation	COSMIC ID	Base change	FFPE Mutant percentage	Plasma Copy per ml
18	G719A	6239	c.2156G>C	5.09%	794
	G719S	6252	c.2155G>A	35.00%	149
	G719C	6253	c.2155G>T	1.10%	161
19	Deletions	26038	c.2233_2247del15	2.02%	100
		13550	c.2235_2248>AATTC	0.61%	75
		6223	c.2235_2249del15	0.54%	75
		6225	c.2236_2250del15	0.74%	75
		18427	c.2237_2257>TCT	1.00%	75
		6220	c.2238_2255del18	0.22%	75
		12367	c.2237_2254del18	0.80%	75
		12384	c.2237_2255>T	0.36%	75
		12678	c.2237_2251del15	0.25%	75
		13551	c.2235_2252>AAT	0.65%	75
		13552	c.2235_2251>AATTC	1.00%	75
12386	c.2237_2252>T	0.78%	75		

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Table 8. Summary of the limit of detection results for the thescreen EGFR Plus RGQ PCR Kit (cont'd)

Exon	Mutation	COSMIC ID	Base change	FFPE Mutant percentage	Plasma Copy per ml
19	Deletions	12416	c.2237_2253>TTGCT	0.60%	75
		12728	c.2236_2253del18	0.79%	75
		12422	c.2238_2248>GC	0.79%	100
		12382	c.2239_2248TTAAGAGAAG>C	2.99%	589
		6218	c.2239_2247delTTAAGAGAA	35.00%	1100
		12387	c.2239_2258>CA	0.93%	75
		12370	c.2240_2257del18	0.77%	75
		12403	c.2239_2256>CAA	0.92%	75
		6255	c.2239_2256del18	0.16%	75
		12383	c.2239_2251>C	0.72%	75
		12419	c.2238_2252>GCA	0.40%	75
		6210	c.2240_2251del12	0.92%	75
		23571	c.2238_2252del15	1.10%	75
		12369	c.2240_2254del15	0.78%	75
		13556	c.2253_2276del24	0.89%	75
12385	c.2235_2255>AAT	0.71%	75		

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Table 8. Summary of the limit of detection results for the *therascreen* EGFR Plus RGQ PCR Kit (cont'd)

Exon	Mutation	COSMIC ID	Base change	FFPE Mutant percentage	Plasma Copy per ml
20	S768I	6241	c.2303G>T	0.69%	167
	Insertions	12376	c.2307_2308insGCCAGCGTG	0.76%	150
		12378	c.2310_2311insGGT	3.04%	131
		12377	c.2319_2320insCAC	0.64%	51
		13428	c.2311_2312insGCGTGGACA	0.76%	100
		13558	c.2309_2310AC>CCAGCGTGGAT	0.89%	100
		T790M	6240	c.2369C>T	1.80%
	C797Sa	6493937	c.2389T>A	1.11%	126
	C797Sb	5945664	c.2390G>C	0.62%	125
	21	L858R	6224	c.2573T>G	0.75%
L861Q		6213	c.2582T>A	0.65%	43

DNA input

The optimized gDNA input to be used in combination with the *therascreen* EGFR Plus RGQ PCR Kit was evaluated on EGFR Positive FFPE samples for 9 EGFR targets (T790M, L861Q, G719A, G719C, G719S, L858R, C797Sa, C797Sb, and S768I) (3 different gDNA inputs, 10 measurements per input sample, 1 *therascreen* EGFR Plus RGQ PCR Kit lot used). The results showed that the optimized input to be used is 25 ng (5 ng/μl).

The optimized ccDNA input to be used in combination with the *therascreen* EGFR Plus RGQ PCR Kit was not evaluated on Plasma samples.

Repeatability

The repeatability was determined on one EGFR positive and one negative FFPE and plasma samples. For each EGFR assay, the repeatability was assessed on a given EGFR mutation, tested on 2 levels of mutation (medium and low). Each level was tested in duplicate on at least 43 runs performed over 20 days, with a minimum of 78 measurements per mutation level and per assay (3 Rotor-Gene Q MDx 5plex HRM instruments, 3 operators, 3 *therascreen* EGFR Plus RGQ PCR Kit lots used). The quantitative analysis of the repeatability results are summarized in Table 9 for FFPE samples and in Table 10 for plasma samples.

Table 9. Summary of the repeatability results for the theascreen EGFR Plus RGQ PCR Kit on FFPE samples

EGFR Plus Mix	EGFR Plus target	Tested Mutation level	Between operator		Between instrument		Between kit lot		Between day		Between run		Within run		Total	
			SD*	CV**	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
T790M_L861Q	T790M	low	0.04	0.51	0.11	1.31	0.00	0.00	0.00	0.00	0.34	4.13	0.17	2.08	0.4	4.84
		medium	0.05	1.03	0.14	2.63	0.00	0	0.00	0.00	0.34	6.41	0.18	3.43	0.41	7.8
	L861Q	low	0.00	0.00	0.36	8.14	0.00	0.00	0.00	0	0.34	7.63	0.26	5.9	0.56	12.62
		medium	0.00	0.00	0.23	11.27	0.03	1..27	0.00	0	0.35	16.92	0.21	10.02	0.47	22.7
	Wild-Type	N/A	0.00	0.00	0.00	0.00	0.20	0.81	0.00	0	0.4	1.59	0.11	0.45	0.46	1.84
	Ins_G719X	Insertions	low	0.15	3.10	0.23	4.55	0.30	6.00	0.00	0	0.64	12.93	0.35	6.97	0.83
medium			0.00	0.00	0.23	11.95	0.21	10.79	0.29	15.29	0.49	25.23	0.17	8.59	0.67	34.69
G719X		low	0.00	0.00	0.52	9.14	0.48	8.49	0.55	9.7	0.78	13.78	0.27	4.74	1.21	21.5
		medium	0.00	0.00	0.49	13.33	0.48	12.82	0.52	14.06	0.63	17.02	0.33	8.84	1.12	30.13
Wild-Type		N/A	0.00	0.00	0.27	1.05	0.28	1.12	0.21	0.81	0.72	2.83	0.18	2.83	0.86	3.39
L858R_C797S		L858R	low	0.00	0.00	0.41	5.76	0.21	2.93	0.43	6.02	0.23	3.25	0.41	5.74	0.79
	medium		0.16	3.40	0.38	8.28	0.00	0.00	0.45	9.72	0.24	5.32	0.38	8.29	0.76	16.48
	C797S	low	0.00	0.00	0.52	9.13	0.24	4.19	0.00	0	0.22	3.82	0.31	5.35	0.69	12
		medium	0.00	0.00	0.35	11.31	0.29	9.23	0.26	8.5	0.36	11.72	0.21	6.69	0.67	21.62
	Wild-Type	N/A	0.20	0.79	0.29	1.11	0.15	0.59	0.44	1.72	0.4	1.58	0.21	0.83	0.74	2.92
	Del_S768I	Deletions	low	0.17	3.10	0.16	2.85	0.00	0.00	0.00	0	0.39	6.95	0.24	4.41	0.51
medium			0.20	5.91	0.24	7.14	0.00	0.00	0.00	0	0.42	12.64	0.15	4.53	0.54	16.31
S768I		low	0.06	0.74	0.35	4.43	0.35	4.43	0.18	2.32	0.42	5.36	0.25	3.2	0.72	9.18
		medium	0.15	2.58	0.27	4.64	0.34	5.82	0.32	5.38	0.31	5.25	0.24	4.17	0.68	11.66
Wild-Type		N/A	0.00	0.00	0.14	0.56	0.28	1.12	0.26	1.02	0.32	1.26	0.15	0.61	0.54	2.13

* SD: Standard deviation

** %CV: Coefficient of variation

Table 10. Summary of the repeatability results for the theascreen EGFR Plus RGQ PCR Kit on plasma samples

EGFR Plus Mix	EGFR Plus target	Tested Mutation level	Between operator		Between instrument SD		Between kit lot		Between day		Between run		Within run		Total	
			SD*	%CV**	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV	SD	%CV
T790M_L861Q	T790M	low	0.1	0.28	0	0	0.16	0.46	0	0	0.29	0.8	0.45	1.27	0.57	1.6
		medium	0.16	0.49	0	0	0.12	0.36	0	0	0.26	0.79	0.24	0.73	0.41	1.23
	L861Q	low	0.24	0.76	0.3	0.96	0.2	0.63	0.38	1.23	0.33	1.06	0.29	0.94	0.72	2.33
		medium	0.14	0.49	0.27	0.93	0.18	0.63	0.18	0.63	0.34	1.18	0.18	0.63	0.55	1.93
	Wild-Type	N/A	0.22	0.84	0.11	0.41	0.32	1.22	0	0	0.42	1.63	0.15	0.57	0.6	2.31
	Ins_G719X	Insertions	low	0.15	0.48	0.08	0.26	0.14	0.46	0.05	0.15	0.1	0.32	0.31	0.99	0.4
medium			0.13	0.43	0	0	0.06	0.22	0.1	0.35	0	0	0.16	0.55	0.24	0.81
G719X		low	0.53	1.84	0.2	0.71	0.14	0.47	0.52	1.8	0.68	2.35	0.15	0.51	1.05	3.62
		medium	0.13	0.47	0.21	0.76	0.39	1.42	0	0	0.57	2.05	0.13	0.47	0.75	2.69
Wild-Type		N/A	0.33	1.24	0.1	0.4	0.25	0.97	0	0	0.43	1.63	0.16	0.6	0.63	2.31
L858R_C797S		L858R	low	0.19	0.56	0	0	0.1	0.3	0.28	0.82	0.33	1	0.3	0.89	0.57
	medium		0.17	0.55	0	0	0.09	0.3	0.22	0.71	0.3	0.96	0.18	0.57	0.45	1.47
	C797S	low	0.12	0.39	0.32	1.01	0.26	0.82	0.14	0.46	0.11	0.36	0.28	0.89	0.54	1.72
		medium	0.09	0.3	0.28	0.98	0.2	0.7	0	0	0.24	0.83	0.12	0.41	0.45	1.55
	Wild-Type	N/A	0		0.31	1.06	0.31	1.08	0.08	0.28	0.28	0.97	0.23	0.8	0.57	1.99
	Del_S768I	Deletions	low	0.66	1.99	0	0	0	0	0.18	0.54	0.84	2.52	0.28	0.84	1.12
medium			0.46	1.5	0	0	0	0	0	0	0.66	2.16	0.28	0.9	0.85	2.78
S768I		low	0.53	1.66	0.16	0.49	0.33	1.04	0.34	1.06	0.66	2.07	0.14	0.44	0.99	3.11
		medium	0.14	0.45	0.24	0.78	0.25	0.81	0.24	0.77	0.35	1.13	0.12	0.39	0.57	1.87
Wild-Type		N/A	0.47	1.8	0.2	0.78	0.26	1.08	0	0	0.45	1.71	0.14	0.52	0.74	2.83

* SD: Standard deviation

** %CV: Coefficient of variation

A qualitative analysis has been performed on the FFPE and plasma repeatability results and showed that the EGFR mutation detection hit rate is independent from the assay kit batch, the Rotor-Gene Q instrument and the operator.

Reproducibility

The reproducibility was determined on one EGFR positive and one negative FFPE and plasma samples. For each EGFR assay, the repeatability was assessed on a given EGFR mutation, tested on 2 levels of mutation (medium and low). Each level was tested in 5 replicates on at least 75 runs (25 runs per site) performed over at least 5 days, with a minimum of 70 measurements per mutation level and per assay (3 sites, one Rotor-Gene Q MDx 5plex HRM instrument per site, one operator per site, one *therascreen* EGFR Plus RGQ PCR Kit lot used). The quantitative analysis of the reproducibility results are summarized in Table 11 for FFPE samples and in Table 12 for plasma samples.

Table 11. Summary of the reproducibility results for the *therascreen* EGFR Plus RGQ PCR Kit on FFPE samples

EGFR Plus Mix	EGFR Plus target	Tested Mutation level	Within run		Between day		Between site		Total	
			SD*	CV**	SD	%CV	SD	%CV	SD	%CV
T790M_L861Q	T790M	low	0.23	2.7	0.48	5.48	0.12	1.38	0.54	6.26
		medium	0.19	3.42	0.44	7.95	0.13	2.29	0.5	8.95
	L861Q	low	0.22	4.85	0.7	15.48	0.31	6.81	0.79	17.59
		medium	0.21	8.7	0.66	27.6	0	0	0.69	28.93
	Wild-Type	N/A	0.14	0.55	0.62	2.46	0.38	1.53	0.74	2.95
	Ins_G719X	Insertions	low	0.28	5.29	0.21	4.02	0	0	0.35
medium			0.15	7.11	0.15	6.87	0.09	3.99	0.23	10.66
G719X		low	0.25	4	0.2	3.14	0.29	4.56	0.43	6.83
		medium	0.18	4.18	0.22	5.11	0.26	6.01	0.39	8.92
Wild-Type		N/A	0.14	0.54	0.14	0.54	0.15	0.61	0.25	0.97
L858R_C797S		L858R	low	0.27	3.4	0.15	1.92	0.33	4.11	0.45
	medium		0.22	4.23	0.15	2.92	0.31	5.96	0.42	7.87
	C797S	low	0.3	4.97	0.12	2.07	0.12	1.93	0.35	5.72
		medium	0.19	5.23	0.16	4.52	0.2	5.59	0.32	8.89
	Wild-Type	N/A	0.12	0.46	0.21	0.82	0.05	0.18	0.24	0.96
	Del_S768I	Deletions	low	0.24	4.16	0.24	4.16	0.19	3.33	0.37
medium			0.15	4.43	0.11	3.12	0.16	4.65	0.25	7.14
S768I		low	0.26	3.29	0.2	2.54	0.14	1.85	0.35	4.55
		medium	0.21	3.66	0.28	4.76	0.13	2.25	0.37	6.41
Wild-Type		N/A	0.12	0.49	0.11	0.45	0.26	1.02	0.31	1.22

* SD: Standard deviation

** %CV: Coefficient of variation

Table 12. Summary of the reproducibility results for the *therascreen* EGFR Plus RGQ PCR Kit on plasma samples

EGFR Plus Mix	EGFR Plus target	Tested Mutation level	Within run		Between day		Between site		Total	
			SD*	%CV**	SD	%CV	SD	%CV	SD	%CV
T790M_L861Q	T790M	low	0.34	0.93	0.26	0.73	0.14	0.4	0.45	1.25
		medium	0.2	0.58	0.22	0.66	0.24	0.73	0.38	1.14
	L861Q	low	0.35	1.11	0.19	0.6	0.17	0.55	0.43	1.37
		medium	0.19	0.65	0.16	0.56	0.23	0.81	0.34	1.18
	Wild-Type	N/A	0.18	0.67	0.86	3.28	0.47	1.8	1	3.8
	Ins_G719X	Insertions	low	0.29	0.93	0	0	0	0	0.3
medium			0.19	0.65	0	0	0	0	0.2	0.67
G719X		low	0.39	1.3	0.64	2.15	0.85	2.86	1.13	3.81
		medium	0.24	0.87	0.33	1.19	0.25	0.9	0.48	1.72
Wild-Type		N/A	0.19	0.72	0.21	0.82	0.16	0.63	0.33	1.26
L858R_C797S		L858R	low	0.37	1.1	0.35	1.04	0.47	1.38	0.69
	medium		0.17	0.55	0.35	1.12	0.48	1.54	0.62	1.98
	C797S	low	0.29	0.94	0.23	0.74	0.31	0.98	0.48	1.54
		medium	0.2	0.68	0.18	0.63	0.35	1.22	0.44	1.53
	Wild-Type	N/A	0.3	1.04	0.38	1.31	0.34	1.18	0.59	2.05
	Del_S768I	Deletions	low	0.3	0.91	0.38	1.16	0.54	1.62	0.73
medium			0.21	0.69	0.32	1.04	0.52	1.7	0.65	2.11
S768I		low	0.17	0.53	0.27	0.84	0.39	1.21	0.5	1.57
		medium	0.2	0.66	0.17	0.56	0.28	0.92	0.39	1.26
Wild-Type		N/A	0.17	0.65	0.19	0.71	0.3	1.13	0.39	1.49

* SD: Standard deviation

** %CV: Coefficient of variation

A qualitative analysis has been performed on the FFPE and plasma reproducibility results and showed that the EGFR mutation detection hit rate is independent from the site.

Interfering substances

A total of 36 potential interfering substances has been tested on 2 EGFR positive and one EGFR negative FFPE and plasma samples (Table 13). The endogenous potential interferents and exogenous substances that can be found in a sample before the DNA preparation were mixed with the samples at a clinically relevant maximal level. The exogenous potential interferents resulting from the DNA preparation workflows were mixed with the extracted DNA at a worst-case calculated level. Each sample (control and mixed with potential interferent) was tested in 6 replicates, resulting on a total of 51 runs (1 *therascreen* EGFR Plus RGQ PCR Kit lot used). The quantitative analysis has not shown any interfering impact of the tested substances.

Table 13. Potential Interfering substances tested on FFPE samples

Substance tested	Tested concentration
Formalin 4-10%	4.10E-05%
Paraffin wax	4.10E-05%
Deparaffinization solution	4.10E-05%
ATL (QIAamp FFPE Lysis Buffer)	1.30E-05%
Proteinase K	4.00E-05%
RNAse A	1.99E-07%
AL buffer (QIAamp FFPE Lysis Buffer)	1.99E-03%
Ethanol 96-100%	1.99E-03%
AW1 (QIAamp FFPE Wash Buffer)	1.00E-01%
AW2 (QIAamp FFPE Wash Buffer)	1.00E+00%
QSB1 (QIASymphony FFPE Buffer)	4.19E-07%
MBS (QIASymphony FFPE Magnetic Beads Solution)	6.15E-09%
QSW1 (QIASymphony FFPE Wash Buffer)	8.80E-04%
QSW2 (QIASymphony FFPE Wash Buffer)	8.80E-02%
AVE (QIASymphony FFPE Elution Buffer)	5.00E+00%
ATE (QIAamp FFPE Tissue Elution Buffer)	5.00E+00%

Table 14. Potential interfering substances tested on plasma samples

Substance tested	Tested concentration
Buffer ACL (QIAamp Plasma Lysis Buffer)	5.77E-05%
Buffer ACB (QIAMP Plasma Buffer)	2.92E-04%
Buffer ACW1 (QIAamp Plasma Wash Buffer)	1.00E-03%
Buffer ACW2 (QIAamp Plasma Wash Buffer)	1.25E-02%
Ethanol 96-100%	1.25E-01%
Buffer AVE (QIAamp Plasma Elution Buffer)	5.00E+00%
MBS3 (QIAsymphony Plasma Magnetic Beads Solution)	3.48E-05%
Proteinase K	7.49E-05%
QSB4 (QIAsymphony Plasma Buffer)	5.57E-04%
QSW8 (QIAsymphony Plasma Wash Buffer)	1.11E-01%
QSW9 (QIAsymphony Plasma Wash Buffer)	4.62E-01%
QSW10 (QIAsymphony Plasma Wash Buffer)	5.00E+00%
QSE1/QSE2 (QIAsymphony Plasma Elution Buffer)	5.00E+00%
Ethylenediaminetetraacetic acid (EDTA)	3.39E+00 µmol/L
Unconjugated Bilirubin	684 µmol/L
Conjugated Bilirubin	475 µmol/L
Haemoglobin	10 g/L
Triglycerides	16.94 mmol/L
Xylene	684 µmol/L
Chloroform	5.00E+00%

Specificity and cross-reactivity

The specificity and the cross-reactivity of the *therascreen* EGFR Plus RGQ PCR Kit were evaluated by testing the ability of the *therascreen* EGFR Plus RGQ PCR Kit to detect and identify correctly (when applicable) the EGFR mutation presented in Table 1. For the FFPE samples, the study was conducted on all EGFR targeted mutations. Specificity for the plasma samples was assessed on C797Sa and C797Sb targeted mutations. All samples were tested in singlicate per *therascreen* EGFR Plus RGQ PCR Kit lot, 3 *therascreen* EGFR Plus RGQ PCR Kit lots were used. The study showed that all targeted mutations were detected by the expected EGFR assay and no signal was observed with other assays.

The detection by the *therascreen* EGFR Plus RGQ PCR Kit of the non-targeted L858Q rare mutation was also assessed by testing FFPE and plasma samples. This study showed that the L858Q mutation is not detected by the C797S assay and might be detected by the L858R assay at high mutation percentage (FFPE) and high copy number (plasma).

Cross contamination and carry-over

The cross-contamination of the 4 EGFR Plus workflows, i.e. using the 4 DNA preparation methods, was evaluated using different conditions alternating EGFR positive and negative samples. At least 3 runs on each EGFR Plus workflows were performed (1 *therascreen* EGFR Plus RGQ PCR Kit lots was used per workflow) and none of the 4 workflows presented cross-contamination.

During the cross-contamination assessment, the carry-over of the four EGFR Plus workflows was evaluated and showed no carry-over between runs.

In-Use Timeframe

The maximal timeframe between the qPCR plate preparation and the launch of the qPCR run was determined for each EGFR assay on a given EGFR mutation, tested on one low level of

mutation (one Rotor-Gene Q MDx 5plex HRM instrument, 1 operator, 1 *therascreen* EGFR Plus RGQ PCR Kit lot used). The 8 different FFPE positive samples have been tested immediately after qPCR plate preparation and after 3 hours, 6 hours, and 24 hours of storage time at +2°C/+8°C. The maximal acceptable time frame is 24 hours; however, it is recommended to launch the *therascreen* EGFR Plus RGQ PCR Kit qPCR run as soon as possible after preparing the plate (i.e., after loading all samples to be tested).

Clinical Performance

Accuracy: Comparison to the analytical reference method

The study demonstrated high level of concordance between *therascreen* EGFR Plus RGQ PCR Kit and the analytical accuracy method(s) (Reference and discrepancy resolution methods used: PNA qPCR - in Karachaliou et al., 2015 and Mayo-de-las-Casas et al., 2017; Sanger bi-directional sequencing; Next-generation sequencing, *therascreen* EGFR RGQ PCR Kit V2, CE (cat. no. 874111) and *therascreen* EGFR Plasma RGQ PCR Kit, CE (cat. no. 870311).

Results were analyzed to assess the Positive Percent Agreement (PPA), Negative Percent Agreement (NPA), and Overall Percent Agreement (OPA) regarding EGFR mutation status (MT or WT) and EGFR target (mutation identification) for FFPE and plasma samples between *therascreen* EGFR Plus RGQ PCR Kit and respective Reference method and following discrepancy resolution method.

In the study, 170 FFPE samples were tested and 148 gave valid interpretable results (148 sample status and 155 target status).

When the results of *therascreen* EGFR Plus RGQ PCR Kit were compared to the results of the respective Reference method, 4 EGFR sample status (MT or WT) showed discordance. Following discrepancy resolution method analysis, the number of discordant samples status (MT or WT) decreased to one discrepant, false negative sample status. The PPA, NPA, and OPA with the corresponding two-sided 95% confidence intervals (CI) are summarized in Table 15 and Table 16.

Table 15. Analysis of Agreement of overall mutation status per sample - *therascreen* EGFR Plus RGQ PCR Kit and Reference Method comparison for FFPE samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall Percent Agreement	97.30%	93.22%	99.26%
Positive Percent Agreement (sensitivity)	93.65%	84.53%	98.24%
Negative Percent Agreement (specificity)	100.00%	95.75%	100,00%

Table 16. Analysis of Agreement of overall mutation status per sample after discordant investigation for FFPE samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall Percent Agreement	99.32%	96.29%	99.98%
Positive Percent Agreement (sensitivity)	98.33%	91.06%	99.96 %
Negative Percent Agreement (specificity)	100,00%	95.89%	100.00%

When the results of *therascreen* EGFR Plus RGQ PCR Kit were compared to the results of the respective Reference method, 9 EGFR target status showed discordance (Table 17). Following discrepancy resolution method analysis, the number of discordant target status decreased to 3 discrepant, 2 false negative, and 1 false positive target status (Table 18). The PPA, NPA, and OPA with the corresponding two-sided 95% confidence intervals (CI) are summarized in Table 19 and Table 20.

Table 17. Detailed FFPE mutation status per target – *therascreen* EGFR Plus RGQ PCR Kit and Reference Method comparison

	Reference method	WT	T790M	L861Q	Insertions	G719X	L858R	C797S	Deletions	Ex21 MT	Total
<i>therascreen</i> EGFR Plus RGQ PCR Kit											
WT		85	1	-	-	2	-	-	2	1	91
T790M		2	2	-	-	-	-	-	-	-	4
L861Q		-	-	1	-	-	-	-	-	-	1
Insertions		1	-	-	1	-	-	-	-	-	2
G719X		-	-	-	-	1	-	-	-	-	1
L858R		-	-	-	-	-	19	-	-	-	19
C797S		-	-	-	-	-	-	1	-	-	1
Deletions		-	-	-	-	-	-	-	36	-	36
Ex21 MT		-	-	-	-	-	-	-	-	-	-
Total		88	3	1	1	3	19	1	38	1	155

Table 18. Detailed FFPE mutation status per target after discordant investigation

<i>therascreen</i> EGFR Plus RGQ PCR Kit \ Reference and discrepant resolution method	WT	T790M	L861Q	Insertions	G719X	L858R	C797S	Deletions	Ex21 MT	Total
WT	89	-	-	-	2	-	-	-	-	91
T790M	-	4	-	-	-	-	-	-	-	4
L861Q	-	-	1	-	-	-	-	-	-	1
Insertions	1	-	-	1	-	-	-	-	-	2
G719X	-	-	-	-	1	-	-	-	-	1
L858R	-	-	-	-	-	19	-	-	-	19
C797S	-	-	-	-	-	-	1	-	-	1
Deletions	-	-	-	-	-	-	-	36	-	36
Ex21 MT	-	-	-	-	-	-	-	-	-	-
Total	90	4	1	1	3	19	1	36	-	155

Table 19. Analysis of Agreement of overall mutation status per target *therascreen* EGFR Plus RGQ PCR Kit and Reference Method comparison for FFPE samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall percent agreement	94.19%	89.26%	97.31%
Positive percent agreement (sensitivity)	91.04%	81.52%	96.64%
Negative percent agreement (specificity)	96.59%	90.36%	99.29%

Table 20. Analysis of Agreement of overall mutation status per target after discordant investigation for FFPE samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall percent agreement	98.06%	94.44%	99.60%
Positive percent agreement (sensitivity)	96.92%	89.32%	99.62%
Negative percent agreement (specificity)	98.89%	93.96%	99.97%

In the study, 106 Plasma samples were tested and 106 gave valid interpretable results (106 sample status and 121 target status).

When the results of *therascreen* EGFR Plus RGQ PCR Kit were compared to the results of the respective Reference method, 9 EGFR sample status (MT or WT) showed discordance. Following discrepancy resolution method analysis, the number of discordant samples status (MT or WT) decreased to 3 discrepant, 1 false negative, and 2 false positive sample status. The PPA, NPA, and OPA with the corresponding two-sided 95% confidence intervals (CI) are summarized in Table 21 and Table 22.

Table 21. Analysis of Agreement of overall mutation status per sample - *therascreen* EGFR Plus RGQ PCR Kit and Reference Method comparison for Plasma samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall percent agreement	91.51%	84.49%	96.04%
Positive percent agreement (sensitivity)	87.27%	75.52%	94.73%
Negative percent agreement (specificity)	96.08%	86.54%	99.52%

Table 22. Analysis of Agreement of overall mutation status per sample after discordant investigation for Plasma samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall percent agreement	97.17%	91.95%	99.41%
Positive percent agreement (sensitivity)	97.96%	89.15%	99.95%
Negative percent agreement (specificity)	96.49%	89.89%	99.57%

When the results of *therascreen* EGFR Plus RGQ PCR Kit were compared to the results of the respective Reference method, 18 EGFR target status showed discordance (Table 23). Following discrepancy resolution method analysis, the number of discordant target status decreased to 5 discrepant, 3 false positive, and 2 false negative target status (Table 24). The PPA, NPA, and OPA with the corresponding two-sided 95% confidence intervals (CI) are summarized in Table 25 and Table 26.

Table 23. Detailed Plasma mutation status per target – *therascreen* EGFR Plus RGQ PCR Kit and Reference Method comparison

<i>therascreen</i> EGFR Plus RGQ PCR Kit \ Reference method	WT	T790M	L861Q	Insertions	L858R	C797S	Deletions	Total
	WT	49	6			6		1
T790M		8						8
L861Q			2					2
Insertions				1				1
L858R					13			13
C797S								0
Deletions	4					1	30	35
Total	53	14	2	1	19	1	31	121

Table 24. Detailed Plasma mutation status per target after discordant investigation

<i>therascreen</i> EGFR Plus RGQ PCR Kit \ Reference and discrepant resolution method	WT	T790M	L861Q	Insertions	L858R	C797S	Deletions	Total
	WT	60	1			1		
T790M		8						8
L861Q			2					2
Insertions				1				1
L858R					13			13
C797S								0
Deletions	3						32	35
Total	63	9	2	1	14	0	32	121

Table 25. Analysis of Agreement of overall mutation status per target *therascreen* EGFR Plus RGQ PCR Kit and Reference Method comparison on Plasma samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall percent agreement	85.12%	77.51%	90.94%
Positive percent agreement (sensitivity)	80.60%	69.11%	89.24%
Negative percent agreement (specificity)	90.74%	79.70%	96.92%

Table 26. Analysis of Agreement of overall mutation status per target after discordant investigation for Plasma samples

		Lower limit interval confidence 95%	Upper limit interval confidence 95%
Overall percent agreement	95.87%	90.62%	98.64%
Positive percent agreement (sensitivity)	96.55%	88.09%	99.58%
Negative percent agreement (specificity)	95.24%	86.71%	99.01%

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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

Run invalid due to invalid Positive Control

- | | |
|---|---|
| a) One component of the reaction mix not added | Check that the reaction mix has been prepared correctly.
Check that all components of the qPCR reaction mix have been added.
Repeat the qPCR run. |
| b) Reaction mix degraded | Kit has been frozen and thawed too many times, the kit contents were not stored at -30 to -15°C , or the primer and probe mixes were not protected from light.
Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit.
Repeat the qPCR run. |
| c) Too low volume pipetted: pipetting volume may be incorrect | Check pipetting scheme and setup of the reaction. Check 5 μl control was added.
Check and recalibrate the pipettes before repeating the qPCR run. |
| d) Fault with Rotor-Gene Q MDx instrument | Check instrument maintenance logs.
Repeat the qPCR run. |
| e) Error in Rotor-Gene Q MDx instrument accessories | The 72-Well Rotor may be incorrectly locked.
Repeat the qPCR run. |

Comments and suggestions

- | | |
|---|--|
| f) Strip tube and/or sample ID inversion | Check the pipetting scheme and setup of the reaction.
Repeat the qPCR run. |
| g) Controls missing or loaded in an incorrect position | Ensure that the correct control is loaded in the correct position.
Repeat the qPCR run. |
| h) Insufficient mixing of control samples | The thawing of the controls was incomplete before loading or the mixing of controls with the reaction mix (pipetting up and down) was not performed correctly.
Repeat the qPCR run. |
| i) Inefficient tube closing | The tube was not efficiently capped, leading to evaporation during the qPCR run.
Repeat the qPCR run. |
| j) The storage conditions for one or more kit components did not comply with the instructions given in "Storage conditions" (page 26) | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary. |
| k) The <i>therascreen</i> EGFR Plus RGQ PCR Kit has expired | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary. |
| l) Incorrect amplification curve (artefact) | Check the corresponding curve for unusual curves (i.e., a straight line).
Repeat the qPCR run. |

Comments and suggestions

Run invalid due to amplification in No Template Control

- | | | |
|----|--|---|
| a) | Cross-contamination or contamination of reagents | Always handle samples, kit components, and consumables in accordance with recommended practices to prevent carryover contamination.

Make sure tips are changed between pipetting different reagents or when loading different tubes. Prepare the qPCR reaction mix with dedicated material (pipettes, tips, etc.).

Prepare the qPCR reaction mix and NTC reaction in a dedicated area where no DNA matrices (DNA, plasmid or PCR products) are introduced.

If cross contamination is identified, replace all reagents.

Repeat the qPCR run. |
| b) | Incorrect amplification curve (artefact) | Check the corresponding curve for unusual curves (i.e., a straight line).

Repeat the qPCR run. |
| c) | Strip tube and/or sample ID inversion | Check the pipetting scheme and setup of the reaction.

Repeat the qPCR run. |
| d) | Fault with Rotor-Gene Q MDx instrument | Check instrument maintenance logs.

For example, lens misalignment may lead to higher background or artifact. If lens alignment is not part of your maintenance plan, please contact QIAGEN Technical Services for more information and potential intervention. |

Sample invalid due to no or low amplification in Internal Control

- | | | |
|----|----------------------------------|--|
| a) | Too low sample DNA concentration | The <i>therascreen</i> EGFR Plus RGQ PCR Kit is optimized for a 5 ng/ μ l working concentration (DNA from FFPE) and 5 μ l pure extract of ccfDNA from plasma samples.

Check the concentration (for FFPE).

Repeat the qPCR step for the sample. |
|----|----------------------------------|--|

Comments and suggestions

- b) Poor DNA sample quality
- Check the concentration (for FFPE).
Repeat the qPCR step with an alternative sample (ideally freshly cut).
Repeat the run using a higher sample concentration.
Note: Testing higher concentration of gDNA may enable an earlier exon2 C_T , but will increase the risk of inhibition. Therefore, the C_T exon 2 may be later than with a lower concentration.
- c) Presence of inhibitor in the sample
- Dilute the sample and repeat the run with lower concentration of sample.
Note: Testing a lower concentration may give an earlier exon 2 signal if inhibitors were present in the sample at 5 ng/ μ l.
Re-extract the sample (FFPE) with more sections and repeat the run.
Note: By eluting more sections in the same volume, the inhibitor concentration may be increased when the sample is normalized at 5 ng/ μ l.
- d) Too low volume pipetted: pipetting volume may be incorrect
- Check pipetting scheme and setup of the reaction. Check 5 μ l of sample was added.
Check and recalibrate the pipettes before repeating the qPCR run.
- e) Inefficient tube closing
- The tube was not efficiently capped, leading to evaporation during the qPCR run.
Repeat the qPCR step for the sample.
- f) The storage conditions for one or more kit components did not comply with the instructions given in "Storage conditions" (page 26)
- Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

Comments and suggestions

- g) The *therascreen* EGFR Plus RGQ PCR Kit has expired
Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
- h) Abnormal fluorescence
Do not write on the tubes. Use caution when handling tubes. Wear gloves.
Check visually the PCR reaction tube for the presence of black magnetic particles (originating from automated QIASymphony extraction). Particles may be removed as described in the respective extraction kit handbooks.

Sample invalid due to early amplification of the Internal Control in samples

- a) Too high sample DNA concentration
The *therascreen* EGFR Plus RGQ PCR Kit is optimized for a 5 ng/ μ l working concentration (FFPE).
For gDNA from FFPE: Check DNA concentration. If DNA is not at this concentration, dilute DNA.
For ccfDNA from plasma: Dilute the sample.
Repeat the qPCR step for the sample.
- b) Too high volume pipetted: Pipetting volume may be incorrect
Check pipetting scheme and setup of the reaction. Check 5 μ l sample was added.
Check and recalibrate the pipettes before repeating the qPCR run for the sample.
- c) The amplification curve may be incorrect
Check the corresponding curve for false positives (i.e., a straight curve instead of exponential amplification).
Repeat the qPCR step for the sample.
- d) Abnormal fluorescence
Do not write on the tubes. Use caution when handling tubes. Wear gloves.
Check visually the PCR reaction tube for the presence of black magnetic particles (originating from automated QIASymphony extraction). Particles may be removed as described in the respective extraction kit handbooks.

Comments and suggestions

- e) Fault with Rotor-Gene Q MDx instrument Check instrument maintenance logs.
For example, lens misalignment may lead to higher background. If lens alignment is not part of your maintenance plan, please contact QIAGEN Technical Services for more information and potential intervention.

Sample invalid due to unexpected early Δ CT or CT

- a) Sample invalid — Set up new PCR run to repeat sample paying close attention
CT too low or is to mixing steps.
below cut off range

Quality Control

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

Quality control of the complete kit has been performed on a Rotor-Gene Q MDx 5plex HRM instrument. This kit is manufactured according to ISO 13485 standard. Certificates of analysis are available upon request at www.qiagen.com/support.

Symbols

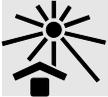
The following symbols may appear in the Instructions for Use or on the packaging and labeling:

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

Symbol definition



Consult instructions for use



Keep away from sunlight



Prescription Use Only



Warning/caution

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 A: Installation of the Rotor-Gene AssayManager v2.1 software, the Gamma Plug-in, and Importing the Assay Profile

Important points before starting

- Rotor-Gene AssayManager software v2.1 must be installed on the computer connected to the Rotor-Gene Q MDx 5Plex HRM instrument. To download the software, go to the Rotor-Gene AssayManager v2.1 product page at www.qiagen.com/9025620 > **Resources** > **Operating Software**. For details about the installation of the Rotor-Gene AssayManager v2.1 Core Application software, refer to the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.
- The *therascreen* EGFR Plus RGQ PCR Kit requires a specific plug-in: the Gamma Plug-in. To access the latest version of this plug-in, go to the Rotor-Gene AssayManager v2.1 product page at www.qiagen.com/9025620 > **Resources** > **Operating Software**. This plug-in must be installed on a computer that already has at least Rotor-Gene AssayManager version 2.1 installed.
- The *therascreen* EGFR Plus RGQ PCR Kit also requires an assay profile. This assay profile contains all parameters needed for PCR cycling and automated data analysis. Two assay profiles are available for use with the *therascreen* EGFR Plus RGQ PCR Kit:
 - One dedicated to the testing of gDNA samples from FFPE :
therascreen_EGFR_Plus_FFPE
 - One dedicated to the testing of ccfDNA samples from plasma:
therascreen_EGFR_Plus_Plasma
- The assay profiles corresponds to “.iap” files that can be downloaded from the *therascreen* EGFR Plus RGQ PCR Kit product page at www.qiagen.com. The assay profiles must be imported in Rotor-Gene AssayManager v2.1 software.

Procedure

Details regarding installation of the Gamma Plug-in and the importing of the assay profiles into the Rotor-Gene AssayManager v2.1 software are as follows.

The installation and importing of the Gamma Plug-in and the assay profile are detailed in the *Rotor-Gene AssayManager v2.1 Core Application User Manual* and the *Gamma Plug-In User Manual*).

1. Download the Gamma Plug-in from the QIAGEN website.
2. To install, double-click the **RGAM_V2_1_Gamma_Plug-in.Installation.V1_0_x.msi** file ($x \geq 0$) and follow the installation instructions. For a detailed description of this process, please refer to the section on “Installing plug-ins” in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.
3. After successful installation of the plug-in, a person with administrator rights for the Rotor-Gene AssayManager v2.1 software needs to import the latest assay profile version as follows.
 - 3a. Go to Windows Explorer, and save the AP in this folder: “
C:\Documents and settings\Public\Documents\QIAGEN\Rotor-Gene AssayManager\Import\AssayProfiles.



The login window appears.

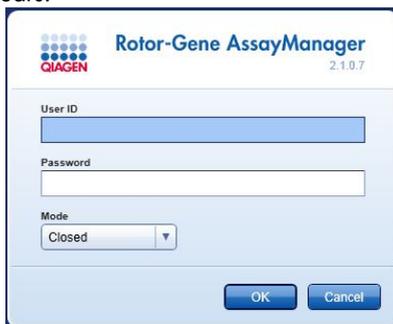


Figure 21. Rotor-Gene AssayManager v2.1.

- 3c. Enter your user ID and password. Leave the **Mode** option as **Closed**. Click **OK**. The Rotor-Gene AssayManager workspace appears.

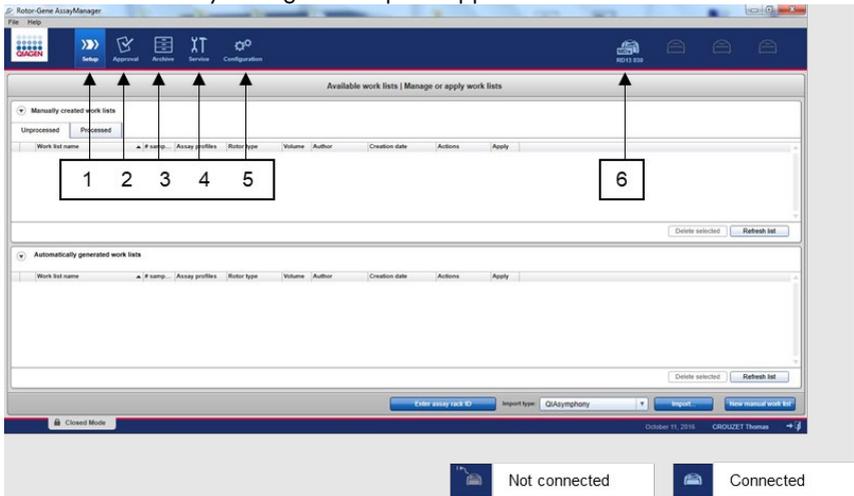


Figure 22. Rotor-Gene AssayManager v2.1. 1 = Setup tab. This tab allows managing or applying work lists 2 = Approval tab. This tab enables you to find previous experiments. 3 = Archive tab. This tab allows you to find previously approved experiments. 4 = Service tab. In this tab, an audit trail of each file generated by the software is reported. 5 = Configuration tab. This tab allows configuration of all software parameters. 6 = Rotor-Gene Q (RGQ) instrument icon; informs user whether a given cycler is connected. Up to four RGQ instruments can be connected to the same computer.

- 3d. For system-wide process safety, perform these required configuration settings for closed mode:
- Select the Settings tab in the Configuration environment.
 - In the Work list panel under Closed mode, check the **Material number required**, **Valid expiry date**, and **Lot number required** boxes.

Note: These configuration settings can only be performed by someone with Administrator rights.

- 3e. In the Configuration environment, select the Assay Profiles tab.
 3f. Click **Import**.
 3g. In the Open file dialog box, select **therascreen_EGFR_Plus_FFPE_V1_0_0.iap** as the first EGFR assay profile.

3h. Click Open.

The assay profile is then loaded and added to the list of available assay profiles that can be used in the Setup environment

3i. Repeat steps 3e–3h to load and add **therascreen_EGFR_Plus_Plasma_V1_0_0.iap** as the second assay profile.

Note: The same version of an assay profile cannot be imported twice.

Appendix B: Running the FFPE and Plasma Assay Profiles in the Same Experiment

Two assay profiles are available use with the *therascreen* EGFR Plus RGQ PCR Kit:

- For testing of gDNA samples from FFPE: **therascreen_EGFR_Plus_FFPE**
- For testing of ccfDNA samples from plasma: **therascreen_EGFR_Plus_Plasma**.

To run both the FFPE and Plasma assay profiles in the same experiment:

1. Set up the qPCR experiment as instructed in “Setting up the qPCR” and “Protocol: Preparing the Rotor-Gene Q MDx 5plex HRM instrument” of “Protocol: *EGFR* mutation assessment by qPCR on Rotor-Gene Q MDx 5Plex HRM instrument” (starting on page 43).



Figure 23) is required when running the two assay profiles in the same experiment:

- Controls (Positive Control, NTC) must be added twice, and positioned in the wells preceding the samples for each sample type (FFPE and plasma), as shown in Figure 23.
- All samples with the same sample type (FFPE or plasma) must be tested in subsequent wells. The order in which the two sample types are tested (i.e. FFPE or plasma first) is not critical.
- There shall not be empty wells between the last well containing a sample from the first sample type (e.g. in Figure 23, well 32 containing sample S6 FFPE) and the first well containing the Positive Control associated with the testing of the second sample type (e.g. in Figure 23, Positive Control in well 33 associated with the Plasma assay profile).

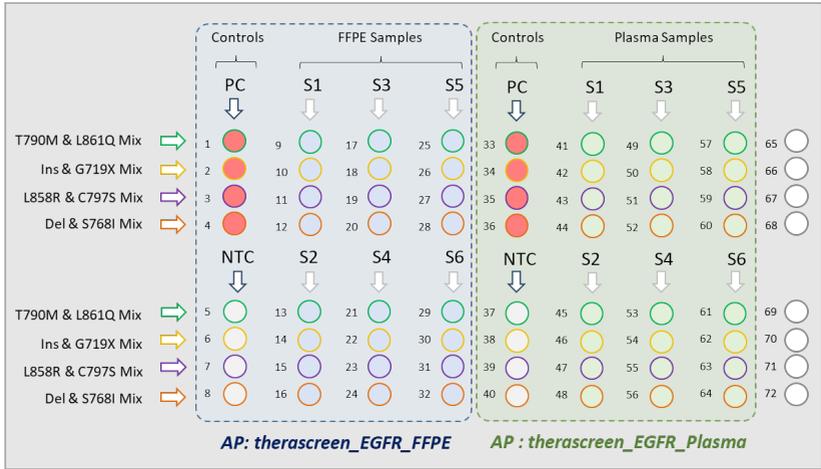


Figure 23. Plate layout to test FFPE and Plasma samples in the same qPCR experiment. PC: EGFRv3 Positive Control; NTC: No template control (water); AP: assay profile; Reaction Mixes: EGFRv3 T790M & L861Q Mix, EGFRv3 Insertions & G719X Mix, EGFRv3 L858R & C797S Mix, EGFRv3 Deletions & S768I Mix. Sample 1 (S1) to Sample 6 (S6): DNA samples. ○ = empty wells.

- Proceed to steps 13–17 of the “Creating a worklist and starting the qPCR run” procedure (starting on page 48).
- Import the two assay profiles successively as instructed in steps 18 and 19 of “Creating a worklist and starting the qPCR run” (page 48). Ensure that the assay profiles are imported in correct order dictated by the plate layout: for instance, if using layout from Figure 23, import the FFPE assay profile first, then the Plasma Assay profile.
Note: The assay profile position can be changed if needed to ensure that the assay profiles are read in the correct order (Figure 24).
- Check the **New strip tube** box to indicate that corresponding assay starts with a new strip tube (Figure 24).

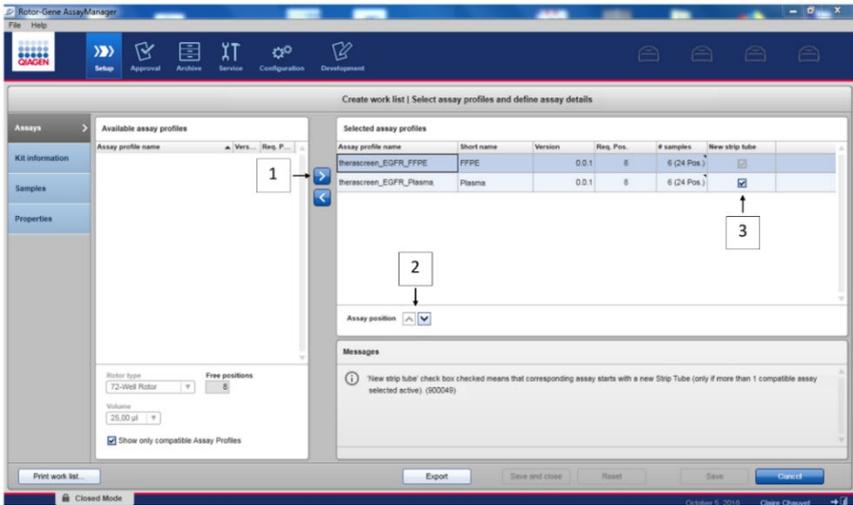


Figure 24. Assay position. 1 = select and transfer the two assay profiles to the worklist. 2 = Assay position can be changed: move assay profile up or down using the arrows. 3 = Check the “New strip tube” box.

5. Continue the procedure from step 20, page 48.

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> EGFR Plus RGQ PCR Kit (24)	For 24 reactions: T790M & L861Q Mix, Insertions & G719X Mix, L858R & C797S Mix, Deletions & S768I Mix, PCR Master Mix, <i>EGFR</i> Positive Control, RNase/DNase-free Water	874611
Rotor-Gene Q and accessories		
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (Green, Yellow, Orange, Red, Crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training included	9002033
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cyclers and High Resolution Melt analyzer with 5 channels (Green, Yellow, Orange, Red, Crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9002032
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipette in 72 x 0.1 ml tubes	9018901
72-Well Rotor	For holding Strip Tubes and Caps 0.1 ml; requires Locking Ring 72-Well Rotor	9018903

Product	Contents	Cat. no.
Locking Ring 72-Well Rotor	For locking Strip Tubes and Caps, 0.1 ml in the 72-Well Rotor	9018904
Rotor Holder	Metal free-standing holder for assembling tubes and Rotor-Discs® into rotors	9018908
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Rotor-Gene AssayManager v2.1	Software for routine testing in combination with Rotor-Gene Q and Rotor-Gene Q MDx instruments	9024203
Rotor-Gene AssayManager v2.1 License (1)	Single license for the installation of Rotor-Gene AssayManager v2.1 software on one computer	9025620
QIAsymphony SP*		
QIAsymphony SP System	QIAsymphony sample prep module: includes installation and training, 1-year warranty on parts and labor	9001751
QIAsymphony SP	QIAsymphony sample prep module: includes 1-year warranty on parts and labor	9001297
Related products		
Deparaffinization Solution (16 ml)	2 x 8 ml Deparaffinization Solution	19093

* For QIAsymphony SP accessories, please refer to the relevant handbooks.

Deparaffinization Solution (50 ml)	1 x 50 ml Deparaffinization Solution	939018
QIAamp DSP DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers and Collection Tubes (2 ml)	60404
QIAamp DSP Circulating NA Kit (50)	For 50 preps: QIAamp Mini columns, Buffers, Carrier DNA, QIAGEN Proteinase K and tubes	61504
QIASymphony DSP DNA Mini Kit (192)	For 192 preps of 200 µl each: Includes 2 reagent cartridges and enzyme racks and accessories	937236
QIASymphony DSP Circulating DNA Kit (192)	Reagent cartridges, accessories and proteinase K vials for 192 preps of 2000 µl or 4000 µl each	937556
RNase A (17,500 U)	2.5 ml (100 mg/ml; 7000 units/ml, solution)	19101
Buffer ATL (4 x 50ml)	4 x 50 ml lysis buffer	939016

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

Revision	Description
R1, March 2022	Initial release

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