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FGFR RGQ RT-PCR Kit

Instructions for Use (Handbook)



Version 1

For research use only

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

REF

8747010



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Intended Use

The FGFR RGQ RT-PCR Kit is a real-time, reverse transcription PCR test for the qualitative detection of two point mutations in exon 7 [p.R248C (c.742C>T) and p.S249C (c.746C>G)], two point mutations in exon 10 [p.G370C (c.1108G>T) and p.Y373C (c.1118A>G)], and three fusions [*FGFR3-TACC3v1*, *FGFR3-TACC3v3*, and *FGFR3-BAIAP2L1*] in the fibroblast growth factor receptor 3 (*FGFR3*) gene, and two fusions [*FGFR2-BICC1* and *FGFR2-CASP7*] in the fibroblast growth factor receptor 2 (*FGFR2*) gene, in RNA samples derived from specimens of formalin-fixed paraffin-embedded (FFPE) urothelial tumor tissue.

Specimens are recommended to be processed using the RNeasy DSP FFPE Kit or RNeasy FFPE kit for manual sample preparation followed by reverse transcription and then automated amplification and detection on the Rotor-Gene Q 5plex HRM instrument.

The test is for research use only.

Summary and Explanation of the Test

Fibroblast growth factor receptors (FGFRs) are tyrosine kinases (transmembrane receptors) present on many types of cell within the body. When activated, FGFRs become phosphorylated at specific tyrosine residues that mediate interaction with cytosolic adaptor proteins and the RAS-MAPK, PI3K-AKT, PLC γ , and STAT intracellular signaling pathways. This signaling pathway is known to play an important role in the control of cell growth, survival, and migration.

The FGFR RGQ RT-PCR Kit is a real-time, reverse transcription PCR test for the qualitative detection of two point mutations in exon 7 [p.R248C (c.742C>T) and p.S249C (c.746C>G)], two point mutations in exon 10 [p.G370C (c.1108G>T) and p.Y373C (c.1118A>G)], and three fusions [*FGFR3-TACC3v1*, *FGFR3-TACC3v3*, and *FGFR3-BAIAP2L1*] in the fibroblast growth factor receptor 3 (*FGFR3*) gene, and two fusions [*FGFR2-BICC1* and *FGFR2-CASP7*] in the fibroblast growth factor receptor 2 (*FGFR2*) gene in RNA samples derived from formalin-fixed paraffin-embedded (FFPE) urothelial tumor tissue specimens.

Specimens are recommended to be processed using the RNeasy DSP FFPE Kit or RNeasy FFPE Kit for manual sample preparation followed by reverse transcription and then automated amplification and detection on the Rotor-Gene Q 5plex HRM instrument.

The test is for research use only.

The *FGFR* alteration targets of the FGFR RGQ RT-PCR Kit assays are detailed in Table 1 and Table 2.

Table 1. FGFR RGQ RT-PCR Kit assays targets: Point mutations

Gene	Amino acid variant	CDS mutation	Cosmic ID	Exons
<i>FGFR3</i>	p.R248C	c.742C>T	COSM714	7
<i>FGFR3</i>	p.S249C	c.746C>G	COSM715	7
<i>FGFR3</i>	p.G370C	c.1108G>T	COSM716	10
<i>FGFR3</i>	p.Y373C	c.1118A>G	COSM718	10

Table 2. FGFR RGQ RT-PCR Kit assay targets: Fusions

Fusion ID	Genes involved	Genomic breakpoints	Exons
<i>FGFR3-TACC3v1</i>	<i>FGFR3</i>	chr4:1808661 C	17
	<i>TACC3</i>	G chr4:1741428	11
<i>FGFR3-TACC3v3</i>	<i>FGFR3</i>	chr4:1808661 C	17
	<i>TACC3</i>	G chr4:1739324	10
<i>FGFR3-BAIAP2L1</i>	<i>FGFR3</i>	chr4:1808661 C	17
	<i>BAIAP2L1</i>	A chr7:97991744	2
<i>FGFR2-BICC1</i>	<i>FGFR2</i>	chr10:123243211 G	17
	<i>BICC1</i>	A chr10:60461834	3
<i>FGFR2-CASP7</i>	<i>FGFR2</i>	chr10:123243211 G	17
	<i>CASP7</i>	A chr10:115457252	2

Principle of the Procedure

The FGFR RGQ RT-PCR Kit is based on the detection by selective amplification of seven *FGFR3* alterations and two *FGFR2* alterations in RNA extracted from FFPE UC specimens, using the Rotor-Gene Q 5plex HRM instrument. Allele-specific technology allows accurate and highly reproducible detection of target *FGFR* alterations, based on the use of specific forward and reverse primer and probe sets; only a perfect match between the primers and probes with the target cDNA allows extension and amplification in the PCR reaction. Analysis of PCR data is automated, with results reported as '**Amplification Detected**' or '-' (when no amplification is detected) for each *FGFR* alteration target.

RNA purification

Formalin fixed, paraffin embedded (FFPE) samples are the most widely available form of solid tumor material. Whilst they are stable and easy to transport and store, the fixation and embedding process damages nucleic acids and mixes them with waxy hydrocarbon materials. Specialist purification techniques must therefore be employed to obtain samples suitable for molecular biological analysis. The RNeasy DSP FFPE Kit has been developed specifically to deal with the challenges posed by RNA purification from FFPE material and we recommend that either this kit, or the functionally-similar RNeasy FFPE Kit are used to prepare RNA that is to be tested using this Kit.

Reverse transcription

To carry out the test procedure, complementary DNA (cDNA) is first synthesized from sample RNA using Reverse Transcriptase. It is this cDNA that then acts as the initial template in an analytical PCR reaction.

Reverse transcription (RT) is performed using a master mix prepared from Reverse Transcriptase enzyme, RT Buffers and RT Primer Mix, which are all provided with the FGFR RGQ RT-PCR Kit. The RT reaction takes place at 42°C and is then stopped by incubation at 95°C.

Reverse Transcriptase is a multifunctional enzyme with three distinct enzymatic activities: an RNA-dependent DNA polymerase, a hybrid-dependent exoribonuclease (RNase H), and a DNA-dependent DNA polymerase. For reverse transcription in vitro, the first two activities are utilized to produce single-stranded cDNA. Firstly, the RNA-dependent DNA-polymerase activity (reverse transcription) transcribes cDNA from an RNA template, forming a DNA:RNA hybrid. Subsequently, the RNase H exonuclease activity specifically degrades only the RNA strand of these hybrids (Figure 1). Therefore, this activity affects RNA hybridized to cDNA, but has no effect on pure RNA. A separate RNA degradation step using RNase H enzyme is not necessary prior to real-time PCR.

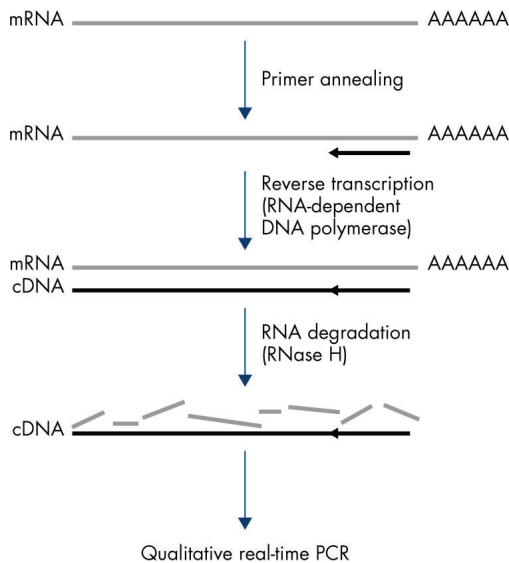


Figure 1. cDNA synthesis. Reverse transcription and first-strand cDNA synthesis.

Real-time PCR

The use of real-time PCR enables the detection of reaction products during the exponential phase of the PCR amplification process, rather than at its end, as is the case with endpoint PCR. This enhances test specificity and reduces the amount of time required to perform the test process.

The assay utilizes the real-time PCR oligonucleotide hydrolysis reaction principle with TaqMan® probes (4). Three types of oligonucleotides are used to detect each target base sequence; a pair of conventional PCR primers, which are complimentary to sequences up- and downstream of the target sequence and form a PCR amplicon, and a probe molecule which is complimentary to the precise target sequence and incorporates both a fluorescent reporter dye, and a fluorescence quencher in close proximity. The 3' end of the probe is a di-deoxy base, to prevent its extension and hence prevent it from acting as another PCR primer in the reaction.

During PCR, if the target sequence of interest is present, the oligonucleotide probe will anneal to it, whilst the PCR primers anneal to their complimentary sequences up- and downstream of the probe binding region. In the primer extension phase of the reaction, the 5' to 3' exonuclease activity of *Thermus aquaticus* (Taq) DNA polymerase cleaves the probe oligonucleotide, freeing both the fluorescent reporter and quencher molecules. As these diffuse away from each other into solution, the effect of the quencher upon the reporter diminishes, leading to an increase in detectable fluorescence. This process occurs in every PCR cycle and does not interfere with the exponential accumulation of product (Figure 2).

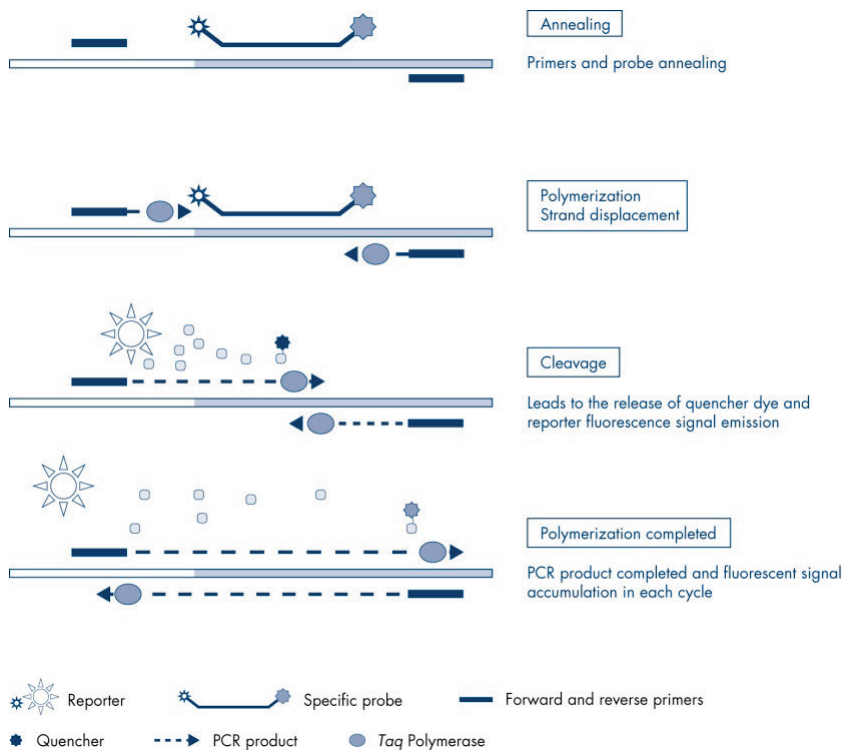


Figure 2. The TaqMan real-time reaction principle.

An increase in fluorescence signal is detected only if the target sequence, which is complementary to both the primers and probe, is present in the reaction. If the target sequence is absent, no probe cleavage will take place, no dissociation of fluorescent reporter from quencher will occur, and hence no increase in fluorescence will be observed. The number of PCR cycles necessary to detect a fluorescent signal above a predetermined threshold is called the cycle threshold (C_T) and is directly proportional to the amount of target present at the beginning of the reaction, allowing a sensitivity limit to be set for the test.

The FGFR RGQ RT-PCR Kit PCR reaction mixes contain HotStarTaq® DNA Polymerase, a modified form of QIAGEN *Taq* DNA Polymerase. It is provided in an inactive state and has no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity. The enzyme is activated at the start of a reaction by a 15-minute, 95°C incubation step. The hot start enables reactions to be set up rapidly and conveniently at room temperature.

The FGFR RGQ RT-PCR Kit PCR reaction mixes also contain a PCR Buffer, which has been specifically developed for multiplex, real-time PCR using sequence-specific probes. This buffer contains a specially optimized combination of KCl and (NH₄)₂SO₄, which promotes a high ratio of specific to nonspecific primer binding during the annealing step of each PCR cycle. This creates stringent primer annealing conditions, leading to increased PCR specificity. When using this buffer, primer annealing is only marginally influenced by the MgCl₂ concentration, so optimization by titration of Mg²⁺ is not required. The buffer also contains synthetic Factor MP, which facilitates multiplex PCR. Factor MP increases the local concentration of primers and probes at the cDNA template and stabilizes specifically bound primers and probes, allowing efficient annealing and extension. The combination of these various components in the PCR Buffer prevents the multiple amplification reactions from interfering with each other.

Platform and Software

The FGFR RGQ RT-PCR Kit is specifically designed for use with the Rotor-Gene Q 5plex HRM instrument installed with the following:

- Rotor-Gene AssayManager® version 2.1
- Gamma Plug-in version 1.0.0
- FGFR FFPE RUO Assay Profile version 1.0.0

Refer to the *Rotor-Gene Q User Manual* for information concerning the Rotor-Gene Q 5plex HRM instrument. The Rotor-Gene Q 5plex HRM instrument must be maintained according to the requirements in the user manual.

Materials Provided

Kit contents

FGFR RGQ RT-PCR Kit		(24)
Catalog no.		8747010
Number of reactions		24
Contents	Cap color	Volume
RT Buffer 1 *	White	200 µl
RT Buffer 2 †	Brown	100 µl
RT Primer Mix	Violet	50 µl
Reverse Transcriptase	Blue	50 µl
Positive Control	Red	100 µl
PC Diluent	Red	200 µl
Mutations-1 Reaction Mix ‡	Orange	720 µl
Mutations-2 Reaction Mix ‡	Violet	720 µl
Fusions-1 Reaction Mix ‡	Yellow	720 µl
Fusions-2 Reaction Mix ‡	Green	720 µl
Water for Sample Dil.	Clear	1.9 ml
Water for NTC	Clear	1.9 ml

* Contains poly(ethylene glycol). For more information, see **General precautions**

† Contains deoxyribonuclease. For more information, **General precautions**

‡ PCR reaction mix containing all the required components, except the sample to be analyzed.

Materials Recommended but Not Provided

Equipment, consumables, and reagents for RNA purification

- RNeasy DSP FFPE Kit (cat. no. 73604) or RNeasy FFPE Kit (cat. no. 73504)
- Calibrated, dedicated pipettes* (adjustable) for sample processing (20 µl, 200 µl and 1 ml)
- RNase and nuclease-free, aerosol-resistant, sterile PCR pipette tips with hydrophobic filters
- Benchtop centrifuge* with rotor for 2 ml tubes
- Vortex mixer*
- Molecular biology grade 100% ethanol†
- Disposable scalpels
- Calibrated heating block capable of incubation between 56°C and 80°C and shaking at 1100 rpm

Equipment and consumables for reverse transcription and real-time PCR

- Calibrated, dedicated pipettes* (adjustable) for sample preparation, master mix preparation, and dispensing of RNA and cDNA (20 µl, 200 µl, and 1 ml)
- RNase and nuclease-free, aerosol-resistant, sterile PCR pipette tips with hydrophobic filters
- 1.5 ml or 2.0 ml nuclease-free, low-binding PCR tubes
- 0.2 ml PCR Tubes (cat. no. 981005)
- 0.1 ml Strip Tubes and Caps (cat. no. 981103 or 981106)

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

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

- Heating block*, water bath*, or thermal cycler* capable of incubating 0.2 ml PCR tubes at 42–95°C
- Cooling system* capable of holding 1.5 ml and 2.0 ml tubes at 0–8°C
- Benchtop centrifuge* with rotor for 0.2 ml, 1.5 ml, and 2.0 ml tubes
- Vortex mixer
- Loading Block 96 x 0.2 ml PCR tubes, aluminum block for manual reaction setup (cat. no. 9018905)
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction setup (cat. no. 9018901)
- 72-Well Rotor (cat. no. 9018903)
- Locking Ring 72-Well Rotor (cat. no. 9018904)
- Rotor Holder (cat. no. 9018908)
- Rotor-Gene Q 5plex HRM Platform (cat. no. 9001580) or Rotor-Gene Q 5plex HRM System (cat. no. 9001650)*
- Rotor-Gene AssayManager software version 2.1
- Rotor-Gene AssayManager Gamma Plug-in version 1.0.0
- FGFR FFPE RUO Assay Profile version 1.0.0

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

Warnings and Precautions

For research use only.

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

For safety information regarding the Rotor-Gene Q 5plex HRM instrument, see the user manual supplied with the instrument.


The FGFR RGQ RT-PCR Kit is to be used by trained personnel in a professional laboratory environment.

For use with the Rotor-Gene Q 5plex HRM instrument.

Recommended for use with the RNeasy DSP FFPE Kit or RNeasy FFPE Kit.

For safety information for the RNeasy DSP FFPE Kit (cat. no. 73604) and the RNeasy FFPE Kit (cat. no. 73504), see the respective Kit handbooks.

An increased NTC invalid run rate of up to approximately 10% has been sporadically observed when using the FGFR RGQ RT-PCR Kit. In the event that an invalid run result is obtained, sample testing should be repeated as described in the Troubleshooting Guide using available sample RNA. If insufficient RNA is available for retest from the original FFPE extraction, re-extraction from FFPE material is required.

<div><div><div>CAUTION</div><div></div></div></div>	<div>DO NOT add bleach or acidic solutions directly to the sample preparation waste.</div>
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The following hazard and precautionary statements apply to components of the FGFR RGQ RT-PCR Kit.

RT Buffer 1



Contains: poly(ethylene glycol). Warning! May cause respiratory irritation. Wear protective gloves/protective clothing/eye protection/face protection.

RT Buffer 2



Contains: deoxyribonuclease. Danger! May cause an allergic skin reaction. May cause allergy or asthma symptoms or breathing difficulties if inhaled. Avoid breathing dust/ fume/ gas/mist/vapors/spray. Wear protective gloves/protective clothing/eye protection/face protection. Wear respiratory protection. If exposed or concerned: Call a POISON CENTER or doctor/physician. Remove person to fresh air and keep comfortable for breathing.

General precautions

Performance of RT-PCR tests requires good laboratory practices, including equipment maintenance, that are appropriate for molecular biology and compliant with applicable regulations and relevant standards.

Note:

- This test was developed for use with clinical FFPE UC specimens.
- All chemicals and biological materials are potentially hazardous and should be treated accordingly. FFPE specimen material and nucleic acids prepared from it are unlikely to pose an infection hazard, but local institutional Health and Safety procedures must always be adhered to.
- Discard sample and assay waste according to your local safety procedures.
- Reagents supplied with the FGFR RGQ RT-PCR Kit are optimally diluted. Diluting reagents may result in a loss of performance.
- The kit is optimized for a reaction volume (reaction mix plus sample) of 25 µl.
- The reagents supplied in the FGFR RGQ RT-PCR Kit are intended to be used only with other reagents from the same kit lot. Substituting any supplied reagents with the same reagents from a different FGFR RGQ RT-PCR Kit production lot may affect performance.
- Refer to the Rotor-Gene Q 5plex HRM instrument user manuals for additional warnings, precautions, and procedures.
- Alteration of incubation times and temperatures may result in erroneous or discordant data.
- Do not use expired or incorrectly stored components.
- Reaction mixes may be degraded if exposed to light.
- Use extreme caution to prevent RNA, cDNA, or PCR product carryover contamination, which may result in a false-positive signal.

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- Use extreme caution to prevent contamination by RNase, which may cause degradation of the template RNA and test failure.
 - The use of individual, dedicated pipettes for setting up reaction mixes and adding templates is recommended.
 - Do not open the Rotor-Gene Q 5plex HRM instrument until the run is finished.
 - Caution must be used to avoid incorrect sample entry, loading errors, and pipetting errors.
 - We recommend that the samples are handled in a systematic way and clearly labeled to ensure correct identification at all times.

We recommend the following precautions are taken:

- Use nuclease-free labware (e.g., pipettes, pipette tips, reaction vials) and wear gloves when performing the assay.
- Use fresh aerosol-resistant pipette tips for all pipetting steps to avoid cross-contamination of the samples and reagents.
- Handle the RT and PCR reagents with dedicated labware (pipettes, tips, etc.) in a dedicated area where no RNA or DNA matrices (RNA, DNA, cDNA, plasmid, or PCR products) are introduced. Add samples to be analyzed in a separate zone (preferably in a separate room or PCR sample preparation cabinet) with dedicated equipment (pipettes, tips, etc.).

Reagent Storage and Handling

Shipping conditions

The FGFR RGQ RT-PCR Kit is shipped on dry ice and must be frozen on arrival. If any component of the FGFR RGQ RT-PCR Kit is not frozen on arrival, the outer packaging has been opened during transit, or the shipment does not contain a packing note, or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Storage conditions

Upon receipt, the FGFR RGQ RT-PCR Kit must be stored immediately at -30 to -15°C in a constant-temperature freezer and protected from light, with the exception of the PC Diluent, which must be immediately removed from the kit box and stored at -90 to -65°C until the stated expiration date.

Stability

Reagents can be stored in their original packaging at -30 to -15°C (with the exception of the PC Diluent, which should always be stored at -90 to -65°C) until the stated expiration date. We recommend that the FGFR RGQ RT-PCR Kit reagents are not subjected to more than five freeze-thaw cycles.

We recommend that the FGFR RGQ RT-PCR Kit reagents are thawed for a minimum of 30 minutes and a maximum of 3 hours before use. Once the reagents are ready to use, the RT or PCR reactions can be set up. We recommend that the total setup time prior to RT or PCR run does not exceed 4 hours.

Specimen Handling and Storage

FFPE specimens

We recommend that RNA samples for testing with the FGFR RGQ RT-PCR Kit are extracted from FFPE UC specimens using either the RNeasy DSP FFPE Kit or the RNeasy FFPE Kit.

RNA samples

Once extracted, RNA samples can either be tested immediately with the FGFR RGQ RT-PCR Kit or stored at -90 to -65°C . We recommend that RNA samples are not subjected to more than five freeze-thaw cycles.

To prepare tissue samples for RNA extraction the following steps are recommended:

- Using a microtome, cut 4–5 μm serial sections from the FFPE sample block and mount them on glass slides.
- A trained individual should assess a Hematoxylin & Eosin (H&E)-stained section to confirm that there is tumor tissue present and mark its boundaries. A slide adjacent to the sections to be extracted is required to identify the tumor surface area and guide macrodissection, if necessary.
- The H&E stained sections must not be used for RNA extraction.
- Store all FFPE blocks and slides at room temperature (15 – 25°C).
- The recommended FFPE input is equivalent to a 4–5 μm slide thickness with a total tumor surface area between 100 mm^2 and 500 mm^2 (inclusive). This can be created by combining material from multiple slides (Figure 3).

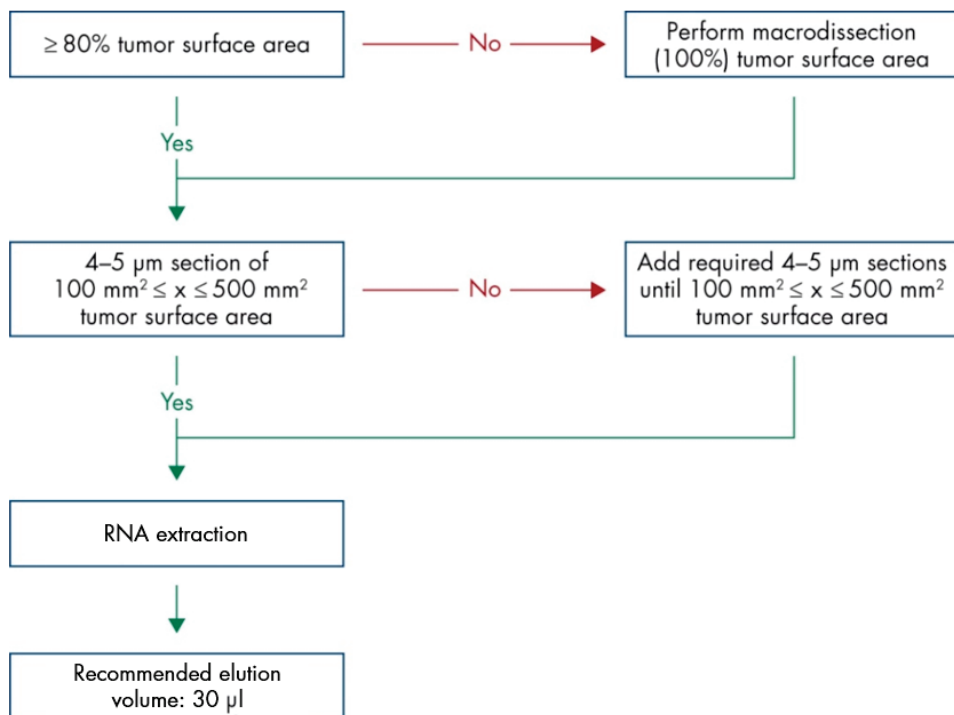


Figure 3. Recommended clinical FFPE UC sample purification workflow to be used with the FGFR RGQ RT-PCR System.

- We recommend that macrodissection is performed to attain a minimum of 80% tumor surface area. If tumor surface area is below 100 mm² and/or the tumor surface area is lower than 80%, additional sections can be used to achieve the recommended sample requirements.

Note: Exercise due care and attention when handling scalpels and ensure that the scalpel blade is pointed away from the body at all times.

Procedure

RNA extraction and preparation

We recommend that RNA is purified using the RNeasy DSP FFPE Kit (cat. no. 73604), or the RNeasy FFPE Kit (cat. no. 73504).

If using the RNeasy FFPE Kit, we recommend that Deparaffinisation Solution (QIAGEN cat. no. 19093) is bought separately and used to deparaffinize samples.

Ensure reagents to be used have not expired and have been transported and stored under the correct conditions.

Important points before starting

- If using the RNeasy DSP FFPE Kit or the RNeasy FFPE Kit for the first time, read the Important Notes section in the *RNeasy DSP FFPE Kit Handbook* or the *RNeasy FFPE Kit Handbook*, as appropriate.
- If working with RNA for the first time, read the Appendix: General Remarks on Handling RNA section in the *RNeasy DSP FFPE Kit Handbook* or *RNeasy FFPE Kit Handbook*.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See the *RNeasy DSP FFPE Kit Handbook* or *RNeasy FFPE Kit Handbook* for safety information.
- Unless otherwise indicated, we recommend that all steps of the procedure are performed at room temperature (15–25°C). During the procedure, work quickly; do not stop in between steps.
- Perform all centrifugation steps using a centrifuge maintained at 15–25°C. If using a refrigerated microcentrifuge, set the instrument to room temperature, otherwise significant cooling below 15°C may occur.

- If using Buffer RPE and RNase-Free DNase I for the first time, reconstitute them as described in the *RNeasy DSP FFPE Kit Handbook* or *RNeasy FFPE Kit Handbook*.
- Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
- Set a heating block with shaking function to 56°C for use in steps 5 and 9. To reduce waiting time, set a second heating block with shaking function to 80°C for use in step 9.

Note: Do not stop the purification procedure in between incubations as increased incubation times might lead to loss or degradation of RNA.

Procedure

- We recommend that the RNA purification procedure as described in the *RNeasy DSP FFPE Kit Handbook* or *RNeasy FFPE Kit Handbook* is followed. In the protocol “Purification of Total RNA from FFPE Tissue Sections”, we recommend that the volumes used for processing >3–4 sections (marked with ●) are followed.
- We recommend that RNA is eluted in 30 µl RNase-Free Water provided with the RNeasy DSP FFPE Kit or RNeasy FFPE Kit.
- We recommend that RNA eluates are stored at –90 to –65°C.

Quantification and normalization of RNA

Procedure

- Blank the spectrophotometer using the RNase-Free Water that was used to elute RNA.
- RNA quantity is determined by measuring optical density at 260 nm.

Note: Total amount of RNA purified = concentration x volume of sample in μl .

- If the RNA concentration is below 16.67 ng/ μl , further processing of the sample is not recommended. Preparing a fresh RNA extraction from a new FFPE specimen is recommended.
- If necessary, dilute the RNA to 16.67 ng/ μl using the Water for Sample Dil. provided in the FGFR RGQ RT-PCR Kit.

Note: The reverse transcription reaction is optimized for 250 ng of purified RNA diluted in a final volume of 15 μl (15 μl x 16.67 ng/ μl = 250 ng RNA input).

Reverse transcription

The FGFR RGQ RT-PCR Kit provides high cDNA yields for sensitive detection of all target transcripts, even those present at low-abundance. Reverse transcription uses an RNA template and a blend of primers complementary to the 3' and 5' ends of the RNA to produce cDNA. Reverse transcriptase (RT) enzyme synthesizes the first-strand cDNA, which is then used as input into the FGFR RGQ RT-PCR Kit PCR reaction.

Important points before starting

- We recommend that all reactions are set up at 0–8°C to minimize the risk of RNA degradation.
- RNase inhibitor and dNTPs are already included in the kit components. It is not necessary to add additional RNase inhibitor and dNTPs.
- We recommend that the RT Primer Mix (supplied with the FGFR RGQ RT-PCR Kit) is used. This RT Primer Mix is optimized to provide high cDNA yields for all regions of RNA transcripts.
- Separate denaturation and annealing steps are not necessary before starting the reverse-transcription reaction.
- After reverse transcription, the reaction should be stopped by incubation at 95°C for 3 minutes, to inactivate the reverse transcriptase.

Procedure

1. Place a loading block capable of holding 96 x 0.2 ml PCR tubes and a cooling block capable of holding 12 or more 2 ml tubes at 0–8°C for at least 60 minutes prior to reaction setup.
2. Thaw RT Buffer 1, RT Buffer 2, Reverse Transcriptase, RT Primer Mix, and Water for NTC provided with the FGFR RGQ RT-PCR Kit at room temperature for between 30 minutes and 3 hours prior to beginning the RT reaction setup.

3. Thaw the test samples, PC, and PC Diluent at 0–8°C for between 30 minutes and 3 hours prior to beginning the RT reaction setup.
4. Inspect all RT reagents closely to ensure that they are completely thawed and dissolved in solution. Dissolve any precipitates in RT Buffer 2 by vortexing. If necessary, briefly incubate the buffer at 37°C until the precipitates dissolve.
5. Pulse vortex all RT reagents 3 times for 3 seconds each time, to ensure reagents are thoroughly mixed.
6. Centrifuge all RT reagents briefly to collect residual liquid from the lids and sides of the tubes.
7. Prepare sufficient reverse transcription master mix for the number of samples to be tested, plus two for the run controls, positive control (PC), and no template control (NTC). The reverse transcription master mix should be prepared at 0–8°C according to Table 3.

Note: We recommend that sufficient master mix is prepared for 2 extra reactions ($n + 2$) if using ≤ 16 samples, and four extra reactions ($n + 4$) if using > 16 samples, to allow sufficient additional volume for the RT setup.

Note: RT Buffer 1 and Reverse Transcriptase are viscous components. It is therefore recommended to pipet them slowly.

Table 3. Reverse transcription reaction mix

Component	Volume per RT reaction
RT Buffer 1 *	5 µl
RT Buffer 2	2.5 µl
RT Primer Mix	1.25 µl
Reverse Transcriptase†	1.25 µl
Total volume of master mix	10 µl

* Includes Mg²⁺ and dNTPs.

† Also contains RNase inhibitor.

8. Pulse vortex the RT reaction mix 3 times for 3 seconds each time, to ensure each reagent is thoroughly mixed.
9. Briefly centrifuge the RT reaction mix to collect residual liquid from the lid and sides of the tube.
10. Store the RT reaction mix in the cooling block.
11. Fill the loading block with the required number of 0.2 ml PCR tubes.
12. Aliquot 10 µl of RT reaction mix into each 0.2 ml tube.
13. Pulse vortex the Water for NTC, PC Diluent, and PC provided with the FGFR RGQ RT-PCR Kit and normalized RNA samples 3 times for 3 seconds each, to ensure each is thoroughly mixed.
14. Briefly centrifuge the Water for NTC, PC Diluent, PC, and normalized RNA samples to collect residual liquid from the lids and sides of the tubes.
15. Add the samples to each tube containing reverse transcription master mix, according to Table 4. Pipet each sample directly into the RT reaction mix of the tube; for the PC sample, pipet the PC first followed by the PC Diluent. After addition of each sample, we recommend to set the pipette to 15 µl, pipet up and down 5–10 times to mix, and immediately cap tubes.

Note: The RT reaction mix and sample (PC, NTC, or test sample) must be mixed thoroughly in order for the RT reaction to proceed efficiently.

Table 4. Sample added to each reverse-transcription reaction mix

Sample name	Sample type	Volume
No template control	Water for NTC	15 µl
Test sample	Sample	15 µl
Positive control	Positive control (PC)	5 µl
	PC Diluent	10 µl

16. Pulse vortex all tubes for 3 seconds to ensure the RT reagents and template are mixed.
17. Ensure that all bubbles are removed and that the RT reagents and template are at the base of the tube.
18. Leave the tubes for 15 minutes in the loading block at room temperature.
19. Incubate the samples in a heating block, water bath, or thermal cycler for 30 minutes at 42°C to reverse transcribe the RNA.
20. Incubate the samples in a heating block, water bath, or thermal cycler for 3 minutes at 95°C to inactivate the Reverse Transcriptase enzyme.
21. Store the cDNA samples until they are used as input for the PCR step. We recommend that samples are stored at 2–8°C for a maximum of 5 days, or at –30 to –15°C for up to 30 days.

Real-time PCR on the Rotor-Gene Q 5plex HRM instrument

The FGFR RGQ RT-PCR Kit provides accurate real-time PCR detection of the specified *FGFR* alterations in a multiplex format. The FGFR RGQ RT-PCR Kit contains four PCR reaction mixes which are provided in a ready-to-use format, including HotStarTaq DNA Polymerase and PCR Buffer.

Procedure

1. Keep a loading block capable of holding 72 x 0.1 ml tubes at 0–8°C for at least 60 minutes prior to reaction setup.
2. Thaw the FGFR RGQ RT-PCR Kit PCR reaction mixes at room temperature for between 30 minutes and 3 hours prior to PCR reaction setup.
3. Pulse vortex all PCR reaction mixes 3 times for 3 seconds each time to ensure each is thoroughly mixed.
4. Centrifuge all PCR reaction mixes briefly to collect residual liquid from the lids and sides of the tubes.
5. Fill the loading block with the required number of 0.1 ml PCR strip tubes.
6. Dispense 20 µl PCR reaction mix into the 0.1 ml PCR strip tubes, according to the pipetting scheme in Figure 4.

72 Well Plate								
	1	2	3	4	5	6	7	8
A	Tube 1 Mut-1	Tube 9 Mut-1	Tube 17 Mut-1	Tube 25 Mut-1	Tube 33 Mut-1	Tube 41 Mut-1	Tube 49 Mut-1	Tube 57 Mut-1
B	Tube 2 Mut-2	Tube 10 Mut-2	Tube 18 Mut-2	Tube 26 Mut-2	Tube 34 Mut-2	Tube 42 Mut-2	Tube 50 Mut-2	Tube 58 Mut-2
C	Tube 3 Fus-1	Tube 11 Fus-1	Tube 19 Fus-1	Tube 27 Fus-1	Tube 35 Fus-1	Tube 43 Fus-1	Tube 51 Fus-1	Tube 59 Fus-1
D	Tube 4 Fus-2	Tube 12 Fus-2	Tube 20 Fus-2	Tube 28 Fus-2	Tube 36 Fus-2	Tube 44 Fus-2	Tube 52 Fus-2	Tube 60 Fus-2
E	Tube 5 Mut-1	Tube 13 Mut-1	Tube 21 Mut-1	Tube 29 Mut-1	Tube 37 Mut-1	Tube 45 Mut-1	Tube 53 Mut-1	Tube 61 Mut-1
F	Tube 6 Mut-2	Tube 14 Mut-2	Tube 22 Mut-2	Tube 30 Mut-2	Tube 38 Mut-2	Tube 46 Mut-2	Tube 54 Mut-2	Tube 62 Mut-2
G	Tube 7 Fus-1	Tube 15 Fus-1	Tube 23 Fus-1	Tube 31 Fus-1	Tube 39 Fus-1	Tube 47 Fus-1	Tube 55 Fus-1	Tube 63 Fus-1
H	Tube 8 Fus-2	Tube 16 Fus-2	Tube 24 Fus-2	Tube 32 Fus-2	Tube 40 Fus-2	Tube 48 Fus-2	Tube 56 Fus-2	Tube 64 Fus-2

Figure 4. Pipetting scheme of PCR reaction mix. Rows A and E (orange): Mutation-1 Reaction Mix. Rows B and F (purple): Mutations-2 Reaction Mix, Rows C and G (yellow): Fusion-1 Reaction Mix. Rows D and H (green): Fusions-2 Reaction Mix. Colors correspond with the PCR tube caps in the kit.

7. Vortex the cDNA samples for 3 seconds, then centrifuge briefly to collect the drops from the lids and sides of the tubes.
8. Add 5 µl of NTC, test sample, or PC from the Reverse Transcription reaction to the 0.1 ml PCR strip tubes, according to the pipetting scheme in Figure 5. Set the pipette to 5 µl and pipet each sample up and down 5–10 times to mix; immediately cap the tubes.

Note: Where possible, the addition of PC cDNA template to the tubes should take place in a different area or laboratory, away from the area or laboratory used to add the NTC and sample cDNA. This is to minimize the potential for sample contamination.

72 Well Plate									
	1	2	3	4	5	6	7	8	9
A	Tube 1 PC	Tube 9 Sample 1	Tube 17 Sample 3	Tube 25 Sample 5	Tube 33 Sample 7	Tube 41 Sample 9	Tube 49 Sample 11	Tube 57 Sample 13	Tube 65 Sample 15
B	Tube 2 PC	Tube 10 Sample 1	Tube 18 Sample 3	Tube 26 Sample 5	Tube 34 Sample 7	Tube 42 Sample 9	Tube 50 Sample 11	Tube 58 Sample 13	Tube 66 Sample 15
C	Tube 3 PC	Tube 11 Sample 1	Tube 19 Sample 3	Tube 27 Sample 5	Tube 35 Sample 7	Tube 43 Sample 9	Tube 51 Sample 11	Tube 59 Sample 13	Tube 67 Sample 15
D	Tube 4 PC	Tube 12 Sample 1	Tube 20 Sample 3	Tube 28 Sample 5	Tube 36 Sample 7	Tube 44 Sample 9	Tube 52 Sample 11	Tube 60 Sample 13	Tube 68 Sample 15
E	Tube 5 NTC	Tube 13 Sample 2	Tube 21 Sample 4	Tube 29 Sample 6	Tube 37 Sample 8	Tube 45 Sample 10	Tube 53 Sample 12	Tube 61 Sample 14	Tube 69 Sample 16
F	Tube 6 NTC	Tube 14 Sample 2	Tube 22 Sample 4	Tube 30 Sample 6	Tube 38 Sample 8	Tube 46 Sample 10	Tube 54 Sample 12	Tube 62 Sample 14	Tube 70 Sample 16
G	Tube 7 NTC	Tube 15 Sample 2	Tube 23 Sample 4	Tube 31 Sample 6	Tube 39 Sample 8	Tube 47 Sample 10	Tube 55 Sample 12	Tube 63 Sample 14	Tube 71 Sample 16
H	Tube 8 NTC	Tube 16 Sample 2	Tube 24 Sample 4	Tube 32 Sample 6	Tube 40 Sample 8	Tube 48 Sample 10	Tube 56 Sample 12	Tube 64 Sample 14	Tube 72 Sample 16

Figure 5. Pipetting scheme of samples tested with the FGFR RGQ RT-PCR Kit. Colors correspond with the PCR tube caps in the kit.

9. Ensure the PCR reaction mix is collected at the base of the 0.1 ml PCR strip tubes.
10. Open the FGFR FFPE RUO Assay Profile version 1.0.0 in the Rotor-Gene AssayManager software version 2.1. See Table 5 for cycling conditions.
11. Place all four strip PCR tubes into the 72-well rotor. Take extra care to ensure that tubes are transferred to the correct positions in 72-well rotor (tube position in 72-well rotor should be the same as tube position in the loading block).

Note: All unused positions on the rotor must be filled with capped, empty tubes. This ensures that the thermal efficiency of the Rotor-Gene Q 5plex HRM instrument is maintained.

12. Place the 72-well rotor into the Rotor-Gene Q 5plex HRM instrument. Ensure that the locking ring (supplied with the Rotor-Gene Q 5plex HRM instrument) is placed on top of the rotor to secure the tubes during the run.
13. To start the real-time PCR run, follow the instructions in "Using Rotor-Gene AssayManager software version 2.1".

Note: The Rotor-Gene Q 5plex HRM instrument cycling conditions for use with the FGFR RGQ RT-PCR Kit are detailed in Table 5.

Table 5. Cycling conditions

Step	Time	Temperature	Additional comments
PCR initial activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step.
2-step cycling*:			
Denaturation	60 sec	94°C	Combined annealing/extension step with fluorescence data collection.
Annealing/extension	90 sec	60°C	
Number of cycles	45		

* Optimal performance is only assured using these cycling conditions.

Using Rotor-Gene AssayManager software version 2.1

Procedure

1. Double-click the Rotor-Gene AssayManager software version 2.1 icon on the desktop of the laptop connected to the Rotor-Gene Q 5plex HRM instrument.
2. The Setup environment appears by default. Click **New manual work list** to create a new work list (Figure 6).

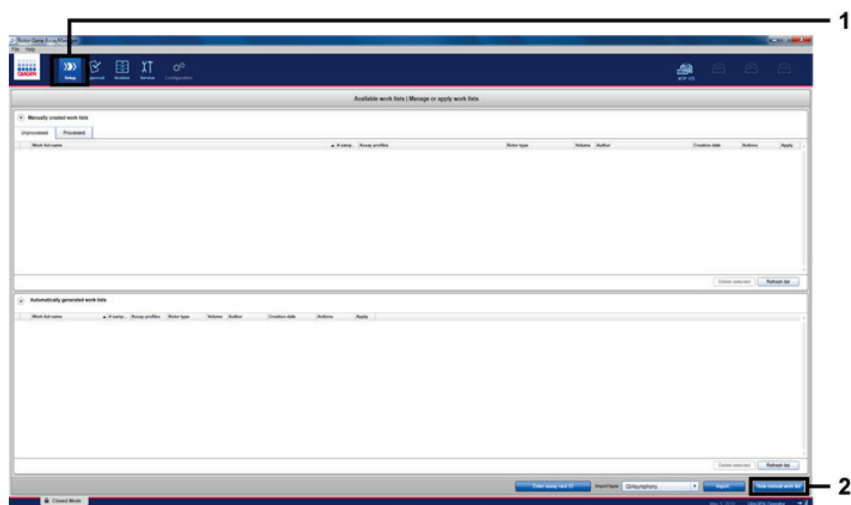


Figure 6. Setting up new manual work list. 1 = Select "Setup" environment, 2 = "New manual work list".

3. Select the Assays tab on the left-hand side of the main window. Select **FGFR FFPE RUO Assay Profile** from the list of available assay profiles and click the blue arrow to move it to the selected assay profiles section. If the assay profile name is truncated, point to the assay profile to see the full name (Figure 7).

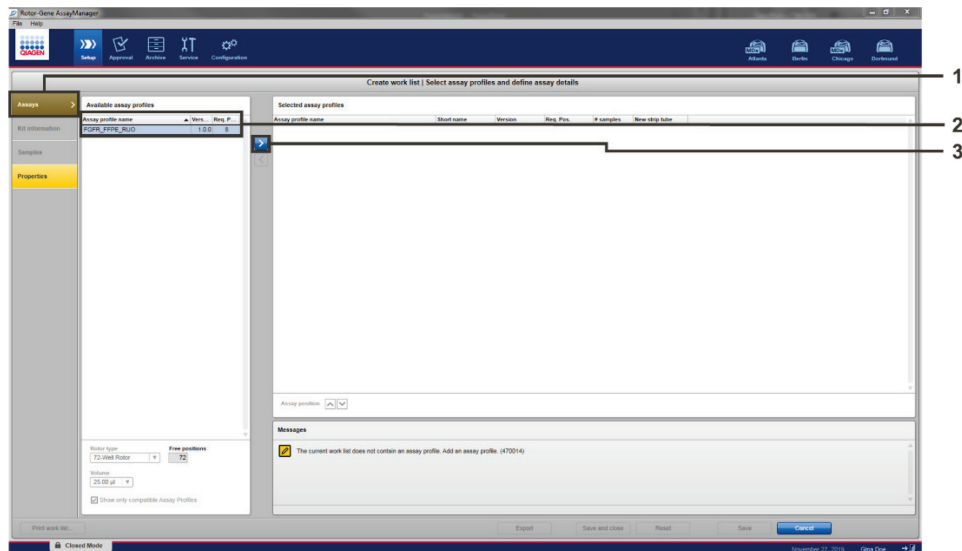


Figure 7. Setting up new manual work list; choosing assay profile name. 1 = "Assays" tab, 2 = Select "FGFR_FFPE_RUO" from the available assay profiles, 3 = Click on the arrow.

4. In the Selected assay profiles window, enter the number of test samples to be tested excluding the number of run controls (Figure 8).

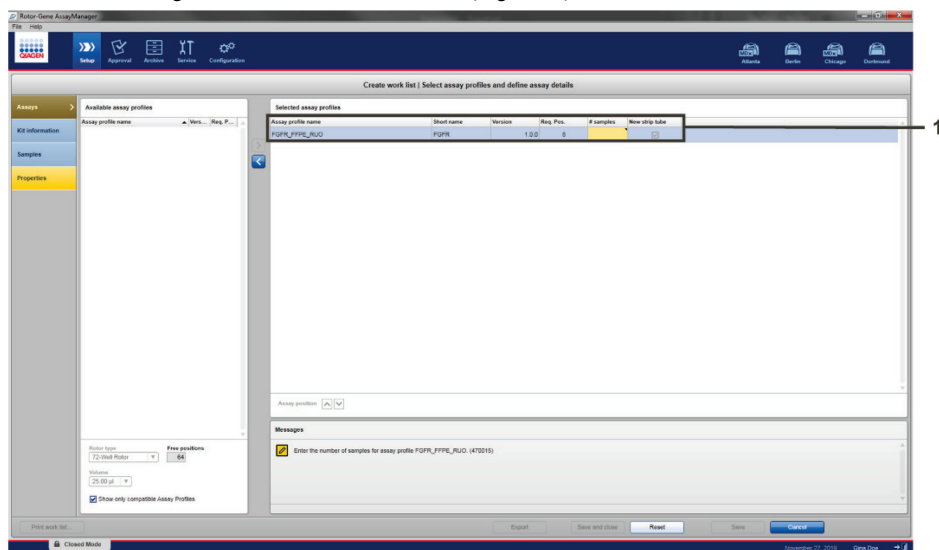


Figure 8. Create work list main window. 1 = Add the number of samples in the "Assay profile name".

5. Click the Kit information tab. Select **Enter kit information manually** and enter the following kit information (Figure 9):

- Material number
- Lot number
- Kit expiry date

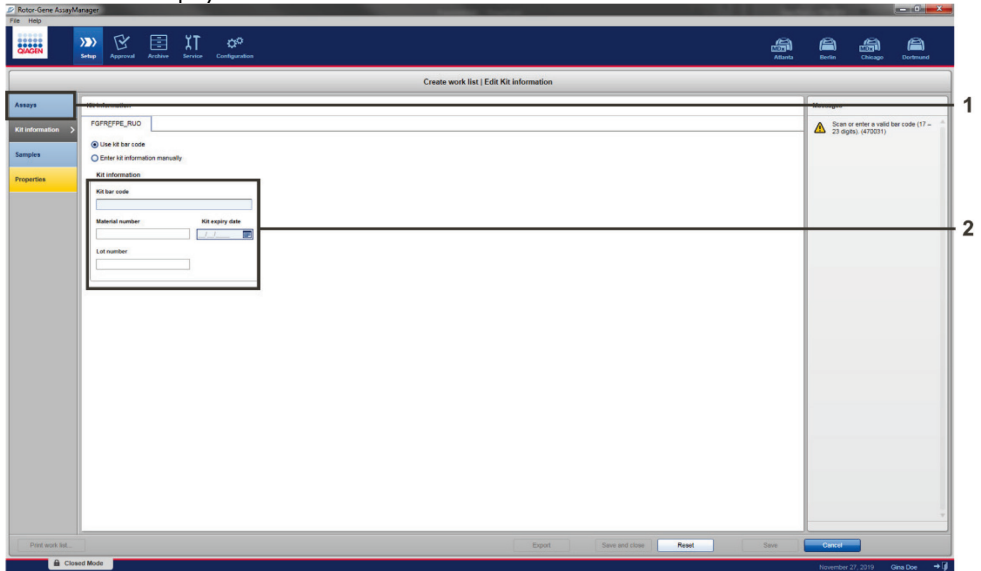


Figure 9. Create work list main window. 1 = "Kit information" tab, 2 = Enter the kit information.

6. Click the Samples tab to enter sample information. Enter the sample names manually (Figure 10).

Note: Ensure correct sample names are entered if using a work list generated for a previous Rotor-Gene AssayManager run.

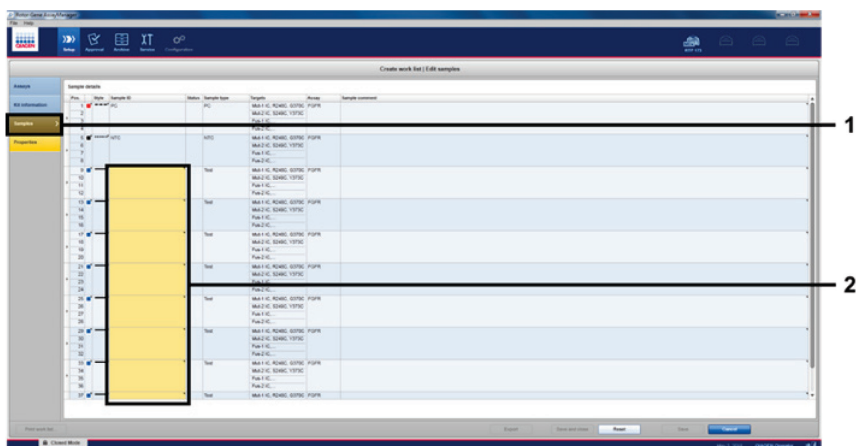


Figure 10. Create work list main window. 1 = "Samples" tab, 2 = Enter sample names.

7. Click the Properties tab and enter the name of the work list. Once the work list name has been entered, ensure that the **is editable** and the **work list is complete** boxes are checked. Then, click **Apply** in the bottom right-hand corner to apply the work list (Figure 11).

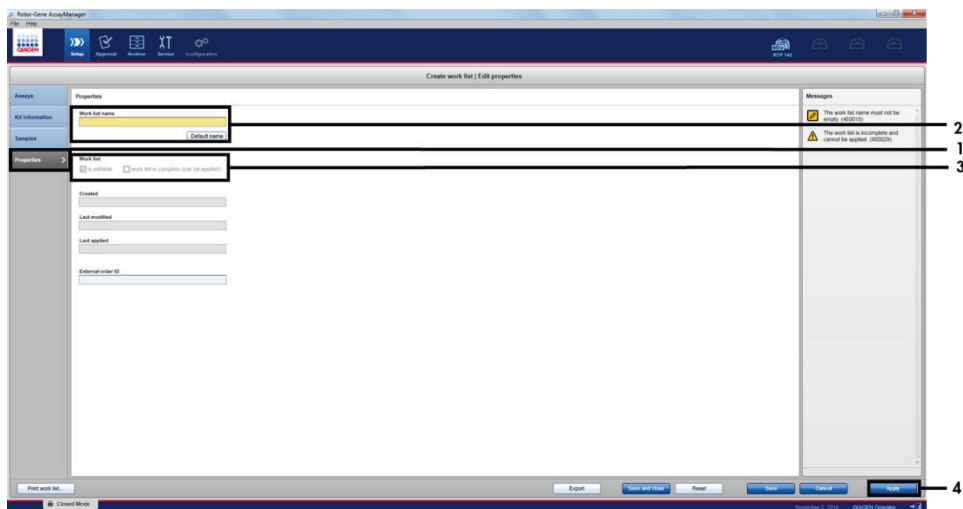


Figure 11. Create work list main window. 1 = "Properties" tab, 2 = Enter work list name, 3 = Select "is editable" and the "work list is complete", 4 = "Apply".

8. Enter the experiment name in the **Experiment name** field. Select a cyclers from the list of available cyclers and ensure that the **Ring attached** box is checked. Once all steps have been performed, click **Start run**. A progress indicator will appear below the Rotor-Gene Q 5plex HRM icon at the top right of the screen to indicate that the run has started (Figure 12).

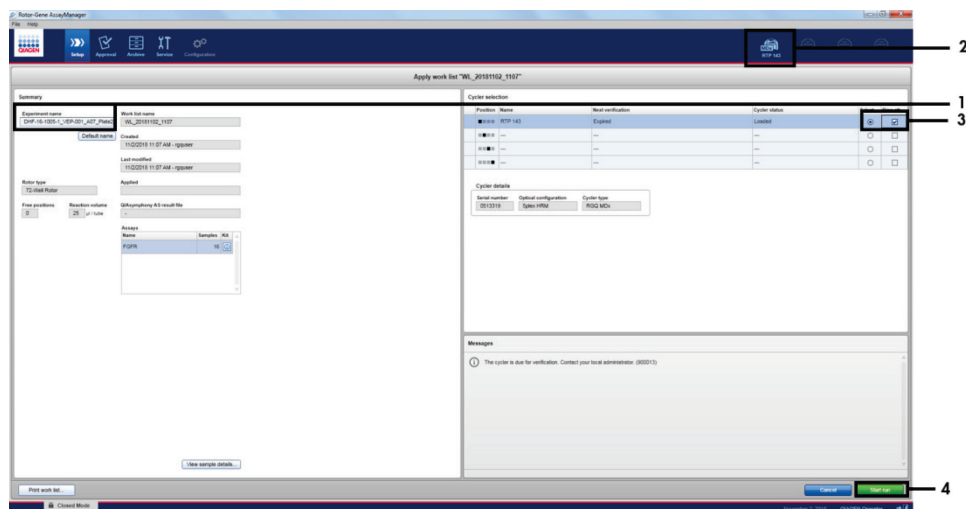


Figure 12. Applying work list and run start. 1 = Enter experiment name, 2 = Current status of instrument, 3 = Select the instrument, 4 = Start run.

Note: The “Cycler” icon changes its appearance depending on the progress and the result of the run (Figure 13). Full descriptions of these cycler icons can be found in the *Rotor-Gene AssayManager v2.1 Core Application User Manual*.

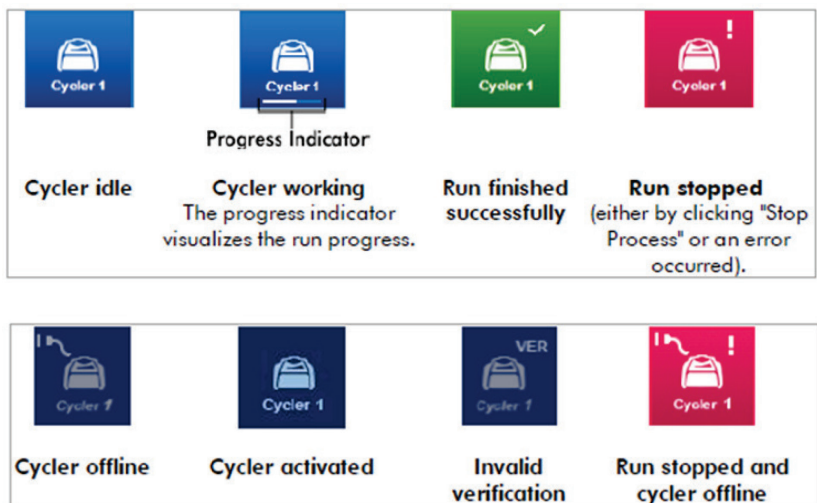


Figure 13. Rotor-Gene Q 5plex HRM Cycler icons that may be displayed.

9. Once the run is complete, click **Finish run**.

The "Finish run" dialog window will open (Figure 14).

Note: During the run process, the amplification curves will be displayed and updated in real time. A progress indicator at the bottom left will show the remaining time.

Important: Do not close down the window when the run is in progress as data loss may occur.

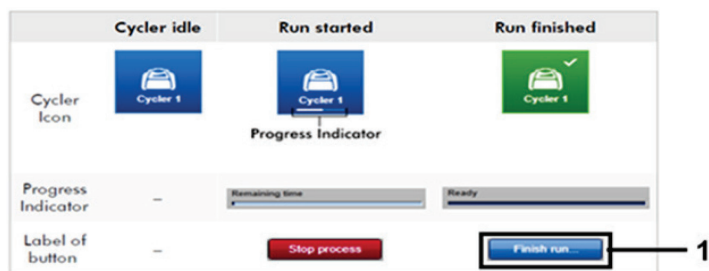


Figure 14. Finishing a run. 1 = "Finish run".

10. Click **Release and go to approval** to enter the Approval environment and release the Rotor-Gene Q 5plex HRM instrument.

The RGQ icon at the upper right of the screen will change from green to blue, indicating that the RGQ instrument is ready to perform another run. Regardless of whether a run is successful or not, the RGQ instrument must be released (Figure 15).

1

Figure 15. "Finish Run" pop up window. 1 = "Release and go to approval".

Note: "Processed data", "Experiment", "Assay", and "Audit trail" information can be found in the "Plots and information" section. Assay results can be found in the "Results" section (Figure 16).

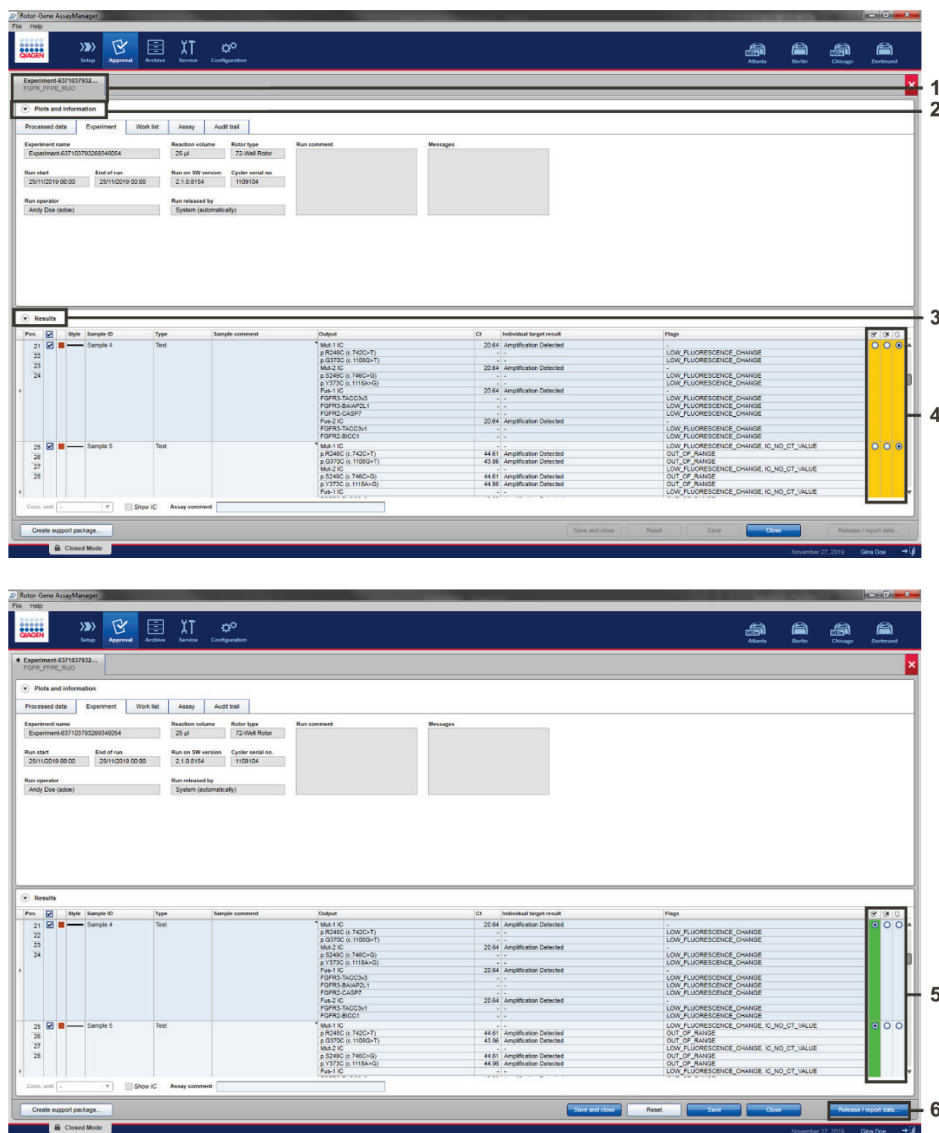


Figure 16. Example of assay results main windows. 1 = "Experiment" tab. 2 = "Plots and information" area. 3 = "Results" area. 4 = "Release/report data". 5 = Accept or reject radio buttons. 6 = Release/report data.

11. Accept or Reject the results of all test samples in the “Results” section of the “Approval” environment.
12. Click **Release/report data**. The Release/report data dialog box will appear, as shown in Figure 17.
13. Click **OK** to save the experiment to the archive and create a LIMS output and run report.

Note: Run reports and LIMS exports will be saved in the default report directory. The default directory can be found in the “Default data export directories” in the “Configuration” section of the software environment.

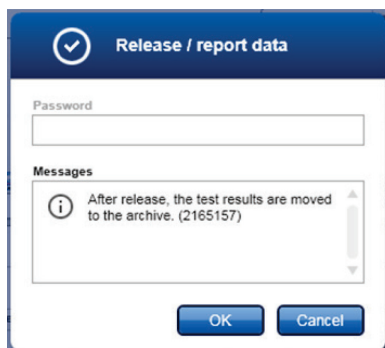


Figure 17. The “Release/report data” dialog box.

14. To view an experiment stored in the experiment archive, click **Archive** software environment and search for the experiment using the search criteria in the Filter Options section. Click **Apply filter** to search. Select an experiment by checking the check box next to the experiment you would like to view and click **Show assays** (Figure 18).

Note: If an experiment fails and an error code is displayed, a list of potential failures and error codes that can be presented by Rotor-Gene AssayManager is contained within the *Rotor-Gene AssayManager v2.1 User Manual* and/or the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

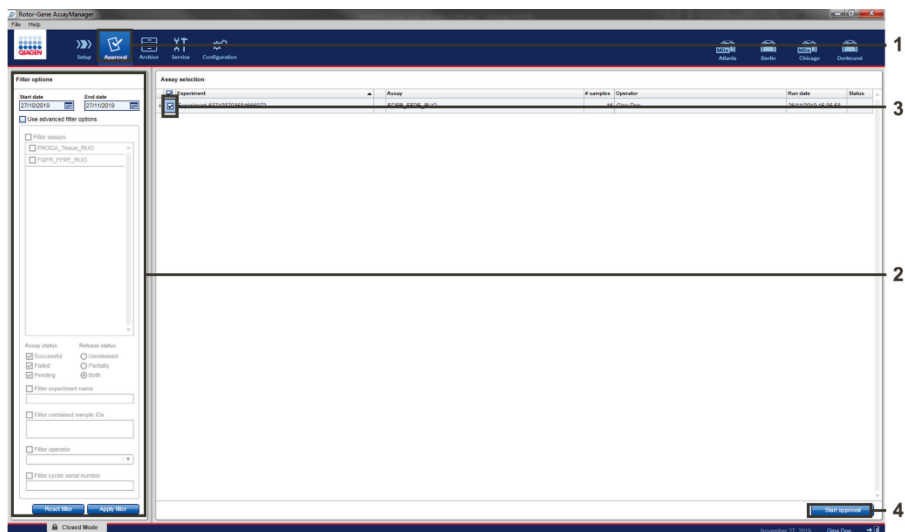


Figure 18. The “Experiment Archive” main window. 1 = “Archive” environment selection, 2 = Search options, 3 = Experiment name selection, 4 = “Show assays” control.

Analysis

Rotor-Gene AssayManager automatically combines all relevant PCR data according to core analysis algorithms defined in the FGFR FFPE RUO assay profile, and reports when amplification is present in a specific reaction, and when the PCR results are outside of the ranges considered normal by QIAGEN.

First, run control analyses are performed:

- The PC reaction is checked for the presence of amplification within the positive control assays and the IC assays. If the C_T value of each PC is outside the relevant PC specifications, an 'OUT_OF_RANGE' warning flag is displayed. The PC specifications considered by QIAGEN to represent optimal FGFR RGQ RT-PCR Kit performance are presented in Table 6. C_T values outside these specifications may indicate incorrect reaction mix set-up.

Table 6. PC Specifications

Target	Lower Limit	Upper Limit
p.R248C (c.742C>T)	22.98	28.58
p.G370C (c.1108G>T)	24.41	30.07
Mut-1 IC	16.33	21.94
p.S249C (c.746C>G)	22.67	30.92
p.Y373C (c.1118A>G)	25.55	34.45
Mut-2 IC	16.24	21.84
FGFR3-TACC3v3	23.39	29.2
FGFR3-BAIAP2L1	22.66	28.26
FGFR2-CASP7	23.23	28.85
Fus-1 IC	16.01	21.62
FGFR3-TACC3v1	22.79	29.85
FGFR2-BICC1	24.55	30.15
Fus-2 IC	15.84	21.44

- The NTC reaction is checked for the absence of specific amplification within the FGFR target assays and the IC assays. If amplification is detected, an 'NTC_OUT_OF_RANGE' warning flag is displayed. The NTC specifications considered by QIAGEN to represent optimal FGFR RGQ RT-PCR Kit performance are presented in Table 7. C_T values outside these specifications may indicate the presence of contamination within the reaction mix.

Table 7. NTC Specifications

Target	Lower Limit	Upper Limit
p.R248C (c.742C>T)	43.00	45.00 or No amplification
p.G370C (c.1108G>T)	43.00	45.00 or No amplification
Mut-1 IC	43.00	45.00 or No amplification
p.S249C (c.746C>G)	39.09	45.00 or No amplification
p.Y373C (c.1118A>G)	43.00	45.00 or No amplification
Mut-2 IC	43.00	45.00 or No amplification
<i>FGFR3-TACC3v3</i>	43.00	45.00 or No amplification
<i>FGFR3-BAIAP2L1</i>	43.00	45.00 or No amplification
<i>FGFR2-CASP7</i>	43.00	45.00 or No amplification
Fus-1 IC	43.00	45.00 or No amplification
<i>FGFR3-TACC3v1</i>	43.00	45.00 or No amplification
<i>FGFR2-BICC1</i>	43.00	45.00 or No amplification
Fus-2 IC	43.00	45.00 or No amplification

If any of these run control flags are displayed, the user may wish to consider repeating the analysis from the reverse transcription step.

Following run control analysis, the internal control analysis is performed on the test samples:

- The IC assays are checked for the presence of amplification across the four PCR reaction mixes. If the C_T value of each IC is outside the relevant specifications, an 'IC_OUT_OF_RANGE' warning flag is displayed. The IC specifications considered by QIAGEN to represent optimal FGFR RGQ RT-PCR Kit performance are presented in Table 8. C_T values outside these specifications may indicate suboptimal RNA quality of the test sample.

Table 8. IC Assay Specifications

Target	Lower Limit	Upper Limit
Mut-1 IC	17.47	25.58
Mut-2 IC	17.47	25.58
Fus-1 IC	17.47	25.58
Fus-2 IC	17.47	25.58

FGFR alteration analysis is then performed on the test samples:

- PCR amplification within the *FGFR* alteration assays is detected. If amplification is detected, **Amplification Detected** is displayed. If no amplification is detected, - is displayed.
- If amplification is detected within an *FGFR* alteration assay but the C_T value is outside the relevant specifications, an 'OUT_OF_RANGE' warning flag is displayed. The assay specifications considered by QIAGEN to be characteristic of detection of an *FGFR* alteration are presented in Table 9. Detection of more than one *FGFR* alteration within a test sample is possible.

Table 9. FGFR Alteration Assay Specifications

Target	Lower Limit	Upper Limit
p.R248C (c.742C>T)	0.00	36.00
p.G370C (c.1108G>T)	0.00	41.00
p.S249C (c.746C>G)	0.00	39.09
p.Y373C (c.1118A>G)	0.00	43.00
FGFR3-TACC3v3	0.00	43.00
FGFR3-BAIAP2L1	0.00	43.00
FGFR2-CASP7	0.00	42.00
FGFR3-TACC3v1	0.00	43.00
FGFR2-BICC1	0.00	43.00

Note: The report generated at the end of the run shows the results obtained from the test samples and run controls, with warning flags displayed in front of any results with 'OUT_OF_RANGE' characteristics. If an error occurs during the Rotor-Gene Q run, the samples in the instrument must be discarded and repeat testing should be performed from the extracted RNA sample.

Note: The report generated at the end of the run shows the results obtained from the test samples and run controls, with warning flags displayed in front of any results with 'OUT_OF_RANGE' characteristics. If an error occurs during the Rotor-Gene Q run, the samples in the instrument must be discarded and repeat testing should be performed from the extracted RNA sample.

All possible flags corresponding to Rotor-Gene AssayManager v2.1 Gamma Plug-in are listed in the Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual. Additional flags which are specific to "FGFR_FFPE_RUO" Assay Profile are shown in Table 10.

Table 10. Example software flags which may be displayed

Software Flags	When this flag is shown	Action to be taken
PC_OUT_OF_RANGE	C _T value is outside the expected range in PC	RT-PCR can be repeated
PC_NO_CT_VALUE	No C _T value in PC	RT-PCR can be repeated
NTC_OUT_OF_RANGE	C _T value detected in NTC	RT-PCR can be repeated
IC_OUT OF_RANGE	C _T value is outside the expected range in sample IC assay	Sample can be retested using the extracted RNA, if available, or re-extracted from fresh FFPE slide
IC_NO_CT_VALUE	No C _T value in sample IC assay	Sample can be retested using the extracted RNA, if available, or re-extracted from fresh FFPE slides
OUT_OF_RANGE	C _T value is above the expected range in sample FGFR alteration assay	Sample can be retested using the extracted RNA or re-extracted from fresh FFPE slides

Limitations of the Procedure

Results from the product are for research use only.

The FGFR RGQ RT-PCR Kit is designed to be used to test RNA derived from specimens of FFPE UC tumors.

The FGFR RGQ RT-PCR Kit is recommended to be used to test RNA prepared using the RNeasy DSP FFPE Kit (cat. no. 73604) or the RNeasy FFPE Kit (cat. no. 73504).

Detection of FGFR alterations is dependent on sample integrity and the amount of amplifiable cDNA that can be derived from the sample.

If the internal control (IC) assay for a particular sample has no C_T value or is outside the specified range, we recommend that the testing procedure be repeated for this sample.

The product is to be used only by personnel specially instructed and trained in suitable laboratory procedures and operation of the Rotor-Gene Q 5plex HRM instrument.

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

Strict compliance with the *FGFR RGQ RT-PCR Kit Instructions for Use (Handbook)* is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended and will result in a loss of performance.

The instructions provided in this handbook are to be used with Rotor-Gene AssayManager software version 2.1, together with the Gamma Plug-in version 1.0.0 and FGFR FFPE RUO Assay Profile version 1.0.0.

The FGFR RGQ RT-PCR Kit does not demonstrate any detectable cross-reactivity between the *FGFR* gene alteration assays it includes.

The FGFR RGQ RT-PCR Kit provides a qualitative test result.

The FGFR RGQ RT-PCR Kit uses a two-step RT-PCR procedure. As with all similar procedures, samples may become contaminated by external sources within the test environment, or potentially by the positive control. Test operators must exercise due caution to avoid contamination of samples and kit reagents.

The effect of microbial contamination upon the performance of the FGFR RGQ RT-PCR Kit is unknown; operators must exercise due caution to avoid introduction of microbial contaminants during testing procedures and should not use kit components if evidence of microbial growth is observed.

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.

Troubleshooting Guide

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

Comments and suggestions

NTC sample shows an 'OUT_OF_RANGE' result

Detection of an assay target has occurred in the absence of template material

Repetition of the test from the RT step using sample RNA is recommended, if this is available. If sample RNA is not available, the test may be repeated from the RNA extraction step.

If possible, close the PCR tubes directly after addition of the sample to be tested.

Make sure that work space and instruments are decontaminated at regular intervals.

Consider repeating the test using new reagents.

PC sample shows an 'OUT_OF_RANGE' result

- | | |
|-------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------|
| a) Incorrect configuration of the RT or PCR | Check your work steps by means of the pipetting scheme and repeat from the RT step. |
| b) The storage conditions for one or more kit components did not comply with the instructions given in Reagent Storage and Handling | Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary. |
| c) The FGFR RGQ RT-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. |

Comments and suggestions

IC shows an 'OUT_OF_RANGE' result

IC is outside the preset expected range; RNA sample quality may be unsuitable for testing with the FGFR RGQ RT-PCR Kit

We recommend that analysis is repeated from the RNA extraction step, if sufficient FFPE tissue is available.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System (ISO 9001), each lot of the FGFR RGQ RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Performance Characteristics

Limit of blank (LoB)

QIAGEN have defined the limit of blank (LoB) for the FGFR RGQ RT-PCR Kit as the upper 95th percentile of results obtained from analysis of *FGFR* alteration-negative samples. The LoB was determined by measuring the levels of breakthrough for each of the nine *FGFR* alteration assays of the FGFR RGQ RT-PCR Kit, where breakthrough is defined as the nonspecific, low-level amplification of an *FGFR* alteration-negative RNA sample. The LoB was determined by the analysis of 60 clinical wild-type samples, using 180 data points per assay, across three FGFR RGQ RT-PCR Kit lots and three instruments. The LoB for each assay within the FGFR RGQ RT-PCR Kit is shown in Table 11.

Table 11. Summary of LoB results

FGFR assay	LoB (C _T value)
p.R248C (c.742C>T)	39.64
p.G370C (c.1108G>T)	No amplification
p.S249C (c.746C>G)	No amplification
p.Y373C (c.1118A>G)	No amplification
FGFR3-TACC3v3	No amplification
FGFR3-BAIAP2L1	No amplification
FGFR2-CASP7	No amplification
FGFR3-TACC3v1	No amplification
FGFR2-BICC1	No amplification

Assay cutoff and cross-reactivity

Assay cutoff

The assay cutoff value is a specific C_T value used to determine whether a sample is classed as positive or negative for an *FGFR* alteration. Samples which generate C_T values at or below the cutoff are classified as *FGFR* alteration-positive (i.e., Amplification Detected, no warning flag) and C_T values generated above the cutoff are classified as *FGFR* alteration-negative (i.e., Amplification Detected with an “OUT_OF_RANGE” flag, or no amplification detected). The false-negative and false-positive rates for each assay were used to determine a cutoff value for each *FGFR* alteration-specific assay, such that a result equal to or less than the cutoff will result in an Amplification Detected (with no “OUT_OF_RANGE” flag) classification. The cutoff for each assay within the FGFR RGQ RT-PCR Kit is shown in Table 12.

Table 12. Summary of assay cutoff results

<i>FGFR</i> assay	Assay cutoff (C_T value)
p.R248C (c.742C>T)	36.00
p.G370C (c.1108G>T)	41.00
p.S249C (c.746C>G)	39.09
p.Y373C (c.1118A>G)	43.00
<i>FGFR3-TACC3v3</i>	43.00
<i>FGFR3-BAIAP2L1</i>	43.00
<i>FGFR2-CASP7</i>	42.00
<i>FGFR3-TACC3v1</i>	43.00
<i>FGFR2-BICC1</i>	43.00

Assay cross-reactivity and analytical specificity

Assay cross-reactivity

Assay cross-reactivity is defined as nonspecific amplification of an *FGFR* alteration by the FGFR RGQ RT-PCR Kit reagents, other than the intended target of an assay, which gives a C_T value below the selected cutoff for that assay. High *FGFR* alteration level samples were tested with the FGFR RGQ RT-PCR Kit and no off-target amplification below the cutoff was observed in any of the assays. Therefore, no cross-reactivity was observed between the assays for *FGFR* alterations that comprise the FGFR RGQ RT-PCR Kit.

Analytical specificity

The level of potential cross-reactivity between primers, probes and blockers used within the FGFR RGQ RT-PCR Kit and non-*FGFR* targets within human and non-human genomes, and the level of potential heterodimer formation was investigated. An *in silico* analysis was performed to determine whether the primers, probes, and blockers used with the FGFR RGQ RT-PCR Kit assays bind nonspecifically within any genome, including the human genome. An additional *in silico* analysis was performed to determine whether the oligonucleotides used within each multiplex assay bind nonspecifically to each other.

The *in silico* analysis of oligo heterodimers indicated that there is a low prevalence of heterodimer formation. The primers and probes do not cross react with either wild-type *FGFR* alleles or any *FGFR* alterations not detected by the FGFR RGQ RT-PCR Kit and therefore will not generate a false signal.

Limit of detection (LoD)

The LoD is defined as the lowest number of *FGFR* alteration RNA copies/ μ l for each target that it is possible to detect 95% of the time. For the *FGFR* alteration-specific assays of the FGFR RGQ RT-PCR Kit, the LoD is reported as *FGFR* alteration RNA copies/ μ l. Individual *FGFR* alteration *in vitro* transcripts, one for each alteration, were spiked into a pool of normalized RNA extracted from wild-type clinical specimens and serially diluted at levels above, at, and below the estimated LoD for each assay.

Sixty replicates of each dilution point in the series were tested using three FGFR RGQ RT-PCR Kit lots. The LoD for each assay was determined using a Probit model and reported as the highest RNA copy number/ μ l (i.e., worst case) LoD detected across the three FGFR RGQ RT-PCR Kit lots tested (Table 13).

Table 13. Summary of LoD results

<i>FGFR</i> assay	LoD (copies/ μ l)
p.R248C (c.742C>T)	75.8
p.G370C (c.1108G>T)	141.57
p.S249C (c.746C>G)	289.82
p.Y373C (c.1118A>G)	274.71
<i>FGFR3-TACC3v3</i>	45.75
<i>FGFR3-BAIAP2L1</i>	9.07
<i>FGFR2-CASP7</i>	27.18
<i>FGFR3-TACC3v1</i>	25.26
<i>FGFR2-BICC1</i>	14.34

Repeatability and reproducibility

The repeatability (within laboratory) of the FGFR RGQ RT-PCR Kit was assessed by testing contrived samples at 3x LoD, representing the nine alterations detected by the FGFR RGQ RT-PCR Kit and an *FGFR* alteration-negative sample.

Repeatability was assessed by testing these samples at one site across multiple days, Rotor-Gene Q instruments, and operators to generate a total of 60 replicates per sample (Table 14).

Table 14. Assay repeatability: Number of correct calls and two-sided 95% confidence limits for each FGFR alteration at 3x LoD and wild-type samples tested across one site

Template	No. correct calls	Percentage of correct calls	Lower two-sided 95% confidence limit	Upper two-sided 95% confidence limit
p.R248C (c.742C>T)	60/60	100%	94.04%	100.00%
p.G370C (c.1108G>T)	60/60	100%	94.04%	100.00%
p.S249C (c.746C>G)	60/60	100%	94.04%	100.00%
p.Y373C (c.1118A>G)	60/60	100%	94.04%	100.00%
<i>FGFR3-TACC3v3</i>	59/60	98.30%	91.06%	99.96%
<i>FGFR3-BAIAP2L1</i>	60/60	100%	94.04%	100.00%
<i>FGFR2-CASP7</i>	60/60	100%	94.04%	100.00%
<i>FGFR3-TACC3v1</i> *	59/60	98.30%	91.06%	99.96%
<i>FGFR2-BICC1</i>	60/60	100%	94.04%	100.00%
WT (Mut-1 Reaction Mix)	60/60	100%	94.04%	100.00%
WT (Mut-2 Reaction Mix)	60/60	100%	94.04%	100.00%
WT (Fus-1 Reaction Mix)	60/60	100%	94.04%	100.00%
WT (Fus-2 Reaction Mix)	59/60	98.3%	91.06%	99.96%

* From 1x LoD samples.

Reproducibility was measured by testing contrived samples at 3x LoD level, clinical samples close to LoD and wild-type samples across the three different sites (one internal QIAGEN site in the United Kingdom and two additional external sites in the USA). The contrived samples for all *FGFR* alterations at 3x LoD and wild-type samples were tested by three operators (per site) over five days using three Rotor-Gene Q instruments at each external site. In addition, RNA extracted from formalin-fixed paraffin embedded (FFPE) UC clinical samples was used to test the device reproducibility. Testing was performed at the LoD level for each target [p.R248C (c.742C>T), p.S249C (c.746C>G), p.G370C (c.1108G>T), p.Y373C (c.1118A>G), *FGFR3-TACC3v1* and *FGFR3-TACC3v3*] utilizing clinical samples. Clinical samples could not be sourced for *FGFR3-BAIAP2L1*, *FGFR2-BICC1*, or *FGFR2-CASP7*. The analysis also combined lot-to-lot variability within the study design. All 1x LoD clinical samples were tested at each of the three sites using the FGFR RGQ RT-PCR Kit (two biological replicates x two FGFR RGQ RT-PCR Kit lots x two operators x three days = 24 replicates at each site). These total replicates were tested across three Rotor-Gene Q instruments at each site, and two of the three FGFR RGQ RT-PCR Kit lots were used alternatively at each site (Table 15).

Table 15. Assay reproducibility: Number of correct calls and two-sided 95% confidence limits for each *FGFR* alteration at LoD, 3x LoD and wild-type samples tested across all sites

Mutation	Target level	Specimen type	No. correct calls results	Percent correct calls	Lower two-sided 95% confidence limit	Upper two-sided 95% confidence limit
p.R248C (c.742C>T)	3x LoD	Contrived	1	100.00%	96.97%	100.00%
	1x LoD	Clinical	1	100.00%	95.01%	100.00%
p.G370C (c.1108G>T)	3x LoD	Contrived	1	100.00%	96.97%	100.00%
	1x LoD	Clinical	71/72	98.61%	92.50%	99.96%
p.S249C (c.746C>G)	3x LoD	Contrived	1	100.00%	96.97%	100.00%
	1x LoD	Clinical	1	100.00%	95.01%	100.00%
p.Y373C (c.1118A>G)	3x LoD	Contrived	1	100.00%	96.97%	100.00%
	1x LoD	Clinical	71/72	98.61%	92.50%	99.96%
<i>FGFR3-TACC3v3</i>	3x LoD	Contrived	119/120	99.17%	95.44%	99.98%
	1x LoD	Clinical	71/72	98.61%	92.50%	99.96%
<i>FGFR3-BAIAP2L1</i> *	3x LoD	Contrived	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	NA	NA	NA	NA
<i>FGFR2-CASP7</i> *	3x LoD	Contrived	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	NA	NA	NA	NA
<i>FGFR3-TACC3v1</i>	1x LoD	Contrived	1	99.17%	95.44%	99.98%
	½x LoD	Clinical	63/72	87.50%	77.59%	94.12%
<i>FGFR2-BICC1</i> *	3x LoD	Contrived	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	NA	NA	NA	NA
WT (Mut-1)	NA	Clinical	120/120	100.00%	96.97%	100.00%
WT (Mut-2)	NA	Clinical	120/120	100.00%	96.97%	100.00%
WT (Fus-1)	NA	Clinical	120/120	100.00%	96.97%	100.00%
WT (Fus-2)	NA	Clinical	116/120	96.67%	91.69%	99.08%

*Clinical UC FFPE samples could not be sourced for these alterations. NA: Not assessed.

Specimen handling

This study assessed sample handling variability, specifically during the RNA extraction step. FFPE UC clinical specimens were divided into three independent sets to be extracted with the RNeasy DSP FFPE Kit at three independent laboratories. Each set contained a predefined number of *FGFR* wild-type and *FGFR* positive (i.e. Amplification Detected) specimens. All specimens were blinded before extraction. Each set was extracted three times by two operators at three sites. All RNA samples were then tested using the *FGFR* RGQ RT-PCR Kit at one site.

Across all *FGFR* alterations, the proportion of correct calls was 96.22%, supporting the reproducibility and repeatability for the *FGFR* RGQ RT-PCR System at the preanalytical step of RNA isolation.

Lot-to-lot interchangeability

This study addressed the potential for lot-to-lot variability to impact *FGFR* alteration detection by the *FGFR* RGQ RT-PCR Kit. The *FGFR* System utilizes two separate kits: the RNeasy DSP FFPE Kit or RNeasy FFPE Kit for isolation of RNA from clinical UC FFPE specimens and the *FGFR* RGQ-RT-PCR Kit for the amplification and detection of *FGFR* alterations.

Lot-to-lot interchangeability was demonstrated using three lots of the RNeasy DSP FFPE Kit and three lots of the *FGFR* RGQ RT-PCR Kit. A combination of clinical and contrived samples was used for this study. Four clinical FFPE specimens harboring the p.R248C (c.742C>T), p.S249C (c.746C>G), *FGFR3-TACC3v1* alterations and a clinical wild-type FFPE specimen were extracted in duplicate using three RNeasy DSP FFPE Kit lots and tested with three different *FGFR* RGQ RT-PCR Kit lots. In addition, contrived samples for the p.G370C (c.1108G>T), p.Y373C (c.1118A>G), *FGFR3-TACC3v3*, *FGFR3-BAIA2PL1*, *FGFR2-BICC1*, and *FGFR2-CASP7* alterations were produced at the 3x LoD level and tested using the three *FGFR* RGQ RT-PCR Kit lots. All samples were normalized and diluted to the 3x LoD level for each assay. A total of 36 replicates for each sample were generated. The overall percentage of correct

calls for all samples across all FGFR RGQ RT-PCR Kit and RNeasy DSP FFPE Kit lots was 99.65% (286/287 samples).

Cross-contamination/analytical carryover

The purpose of this study was to evaluate the FGFR RGQ RT-PCR Kit for carryover when high *FGFR* alteration-positive samples are tested adjacent to *FGFR* alteration-negative samples. The study examined the entire FGFR System from extraction to PCR amplification and investigated if carryover occurred between samples, extractions, and within or between runs.

Clinical UC FFPE samples were divided into two independent sets. Both sets comprised 18 wild-type samples and 12 *FGFR* alteration-positive samples. The RNA extraction, RT and PCR reaction setup followed a matrix designed to introduce the risk of sample cross-contamination. Each set was tested by a different operator using the same FGFR RGQ RT-PCR Kit lot. A total of 128 wild-type replicates were tested and the percentage of false-positive calls for the wild-type samples was 3.13% (4/128 samples).

Interfering substances













This study investigated the impact of potential endogenous and exogenous interferents on *FGFR* alteration status detection across the RNeasy DSP FFPE Kit and the FGFR RGQ RT-PCR Kit. Samples were spiked with one of four interferents (hemoglobin, Buffer RPE, Deparaffinization Solution, or paraffin wax), either during the extraction step or the RNA sample normalization step. A total of 60 replicates per interferent across each of the nine *FGFR* alteration-positive samples and wild-type samples were tested. The four interferents showed no statistically significant difference in results between the control samples and test samples, with the presence of interferents not affecting assay performance or target call.

References

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- Rodriguez-Vida, A., Saggese, M., Hughes, S., et al. (2015) Complexity of FGFR signaling in metastatic urothelial cancer. J. Hematol. Oncol. **24**, 119.
- Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H. (1991) Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **88**, 7276.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	Catalog number
	Lot number
	Contains
	Number
	Protect from light
	Global Trade Item Number
Rn	R is for revision of the Instructions for Use (Handbook) and n is the revision number
	Temperature limitation
	Manufacturer
	Consult instructions for use
	Caution

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Ordering Information

Product	Contents	Cat. no.
FGFR RGQ RT-PCR Kit (24)	For 24 reactions: RT Buffer 1, RT Buffer 2, RT Primer Mix, Reverse Transcriptase, Positive Control, PC Diluent, Mutations-1 Reaction Mix, Mutations-2 Reaction Mix, Fusions-1 Reaction Mix, Fusions-2 Reaction Mix, Water for Sample Dil., Water for NTC	8747010
RNeasy DSP FFPE Tissue Kit		
RNeasy DSP FFPE Kit	For 50 RNA preps: RNeasy MinElute® Spin Columns, Collection Tubes, Elution Tubes, Deparaffinization Solution, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers and RNase-Free Water.	73604
RNeasy FFPE Tissue Kit		
RNeasy FFPE Kit	For 50 RNA preps: RNeasy MinElute Spin Columns, Collection Tubes, Proteinase K, RNase-Free DNase I, DNase Booster Buffer, RNase-Free Buffers, RNase-Free Water	73504
Deparaffinisation Solution	2 x ml Deparaffinisation Solution	19093

Rotor-Gene Q 5plex HRM and accessories

Rotor-Gene Q 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: includes 1-year warranty on parts and labor, installation and training not included	9001580
Rotor-Gene Q 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: includes 1-year warranty on parts and labor, installation and training	9001650
PCR Tubes, 0.2 ml (1000)	1000 thin-walled tubes for 1000 reactions of 20–50 µl	981005
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Loading Block 96 x 0.2 ml PCR Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 96 x 0.2 ml PCR tubes	9018905
72-Well Rotor	For holding Strip Tubes and Caps, 0.1 ml, with reaction volumes of 10–50 µl; requires Locking Ring 72-Well Rotor	9018903
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

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Document Revision History

Date	Changes
December 2019	Initial release

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