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therascreen[®] FGFR RGQ RT-PCR Kit Instructions for Use (Handbook)



Version 2

IVD

For in vitro diagnostic use

Rx only (For prescription use only)

For use with RNeasy[®] DSP FFPE Kit

For use with Rotor-Gene[®] Q MDx (US) instruments

REF

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Intended Use

The *therascreen* 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), p.S249C (c.746C>G)], two point mutations in exon 10 [p.G370C (c.1108G>T) and p.Y373C (c.1118A>G)] and two fusions (FGFR3-TACC3v1 and FGFR3-TACC3v3) in the fibroblast growth factor receptor 3 (FGFR3) gene in RNA samples derived from formalin-fixed paraffin-embedded (FFPE) urothelial tumor tissue. The test is indicated for use as an aid in identifying patients with cases of urothelial cancer (UC) which harbor these alterations and are therefore eligible for treatment with BALVERSA™ (erdafitinib).

Specimens are processed using the RNeasy DSP FFPE Kit for manual sample preparation followed by reverse transcription and then automated amplification and detection on the Rotor-Gene Q MDx (US) instrument.

Summary and Explanation of the Test

Fibroblast growth factor receptors (FGFRs) are tyrosine kinases (transmembrane receptors) present in many types of cell within the body. Activated FGFRs phosphorylate 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 (1) and is therefore an attractive target for cancer therapies.

Activating alterations within the FGFR genes have been found in a subset of UC patients (2, 3), demonstrating the importance of this type of genetic defect as a driver of tumor development and progression.

The *therascreen* 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), p.S249C (c.746C>G)], two point mutations in exon 10 [p.G370C (c.1108G>T) and p.Y373C (c.1118A>G)] and two fusions (FGFR3-TACC3v1 and FGFR3-TACC3v3) in the fibroblast growth factor receptor 3 (FGFR3) gene in RNA samples derived from formalin-fixed paraffin-embedded (FFPE) urothelial tumor tissue. The test is indicated for use as an aid in identifying patients with cases of UC that harbor these alterations and are therefore eligible for treatment with BALVERSA (erdafitinib).

Specimens are processed using the RNeasy DSP FFPE Kit for manual sample preparation followed by reverse transcription and then automated amplification and detection on the Rotor-Gene Q MDx (US) instrument.

The *therascreen* FGFR RGQ RT-PCR kit is also designed to identify FGFR2 fusions FGFR2-BICC1 and FGFR2-CASP7 and FGFR3 fusion FGFR3-BAIAP2L1, because patients harboring these FGFR fusions were eligible for the clinical trial. However, the test is not clinically validated to detect these three fusions. Drug safety and efficacy has not been established for cases of UC

harboring these fusions and no claims are made for the use of the *therascreen* FGFR RGQ RT-PCR kit as an aid in the selection of such patients for treatment with BALVERSA (erdafitinib).

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

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

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

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

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

The test was designed to identify FGFR2 fusions FGFR2-BICC1 and FGFR2-CASP7 and FGFR3 fusion FGFR3-BAIAP2L1 because these FGFR fusion patients were eligible for the trial. However, the QIAGEN test is not clinically validated to detect these three fusions.

Principle of the Procedure

The *therascreen* FGFR RGQ RT-PCR Kit is based on the detection by selective amplification of six FGFR3 alterations in RNA extracted from FFPE UC specimens, using the Rotor-Gene Q MDx (US) system. 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. Result reporting is fully automated. If both the run controls and the sample results are valid and assay target amplification is below the pre-determined cutoff threshold, the report will display the FGFR alteration(s) detected in each sample.

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 in vitro diagnostic analysis. The RNeasy DSP FFPE Kit has been developed specifically to deal with the challenges posed by RNA purification from FFPE material and must be 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 *therascreen* 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.

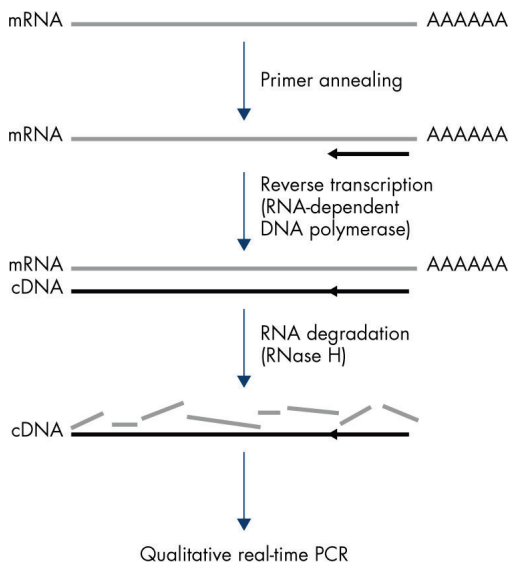


Figure 1. cDNA synthesis. Reverse transcription and first-strand cDNA synthesis.

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).

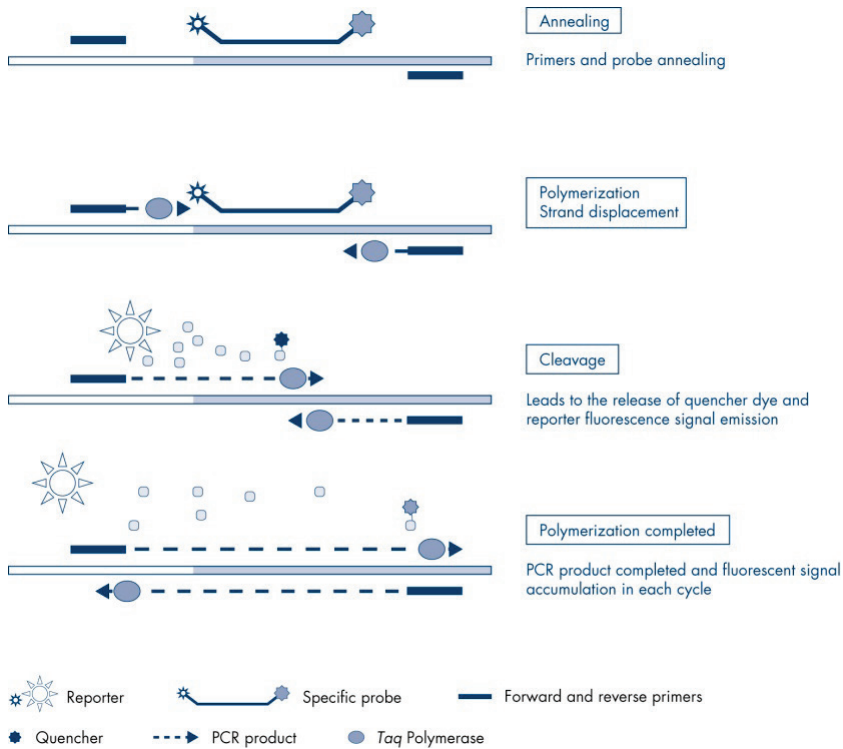


Figure 2. The TaqMan real-time reaction principle.

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 pre-determined 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 *therascreen* 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 *therascreen* 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 *therascreen* FGFR RGQ RT-PCR Kit is specifically designed for use with the Rotor-Gene Q MDx (US) instrument installed with:

- Rotor-Gene AssayManager® version 2.1
- Gamma MDx Plug-in version 1.0.0
- *therascreen* FGFR FFPE MDx Assay Profile version 1.0.3

Refer to the *Rotor-Gene Q MDx User Manual (US)* for information concerning the Rotor-Gene Q MDx (US) instrument. The Rotor-Gene Q MDx (US) instrument must be maintained according to the requirements in the user manual.

Materials Provided

Kit contents

<i>therascreen</i> FGFR RGQ RT-PCR Kit		(24)
Catalog no.		874721
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
<i>therascreen FGFR RGQ RT-PCR Kit Instructions for Use (Handbook)</i>	–	1

* Contains poly(ethylene glycol). For more information, see page 18.

† Contains deoxyribonuclease. For more information, see page 18.

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

Materials Required but Not Provided

Equipment, consumables and reagents for RNA purification

- RNeasy DSP FFPE Kit (cat. no. 73604)
- 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)
- Heating block*, water bath* or thermal cycler* capable of incubating 0.2 ml PCR tubes at 42–95°C

* 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.

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- 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)
 - Rotor-Disc® 72 (cat. no. 9018899)
 - Rotor-Disc 72 Locking Ring (cat. no. 9018900)
 - Rotor Holder (cat. no. 9018908)
 - Rotor-Gene Q MDx Platform (US)* (cat. no. 9002035) or Rotor-Gene Q MDx System (US)* (cat. no. 9002036)
 - Rotor-Gene AssayManager software version 2.1
 - Rotor-Gene AssayManager Gamma MDx Plug-in version 1.0.0
 - *therascreen* FGFR FFPE MDx Assay Profile version 1.0.3

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

Warnings and Precautions

For in vitro diagnostic use

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 MDx (US) instrument, see the user manual supplied with the instrument.

For safety information for the RNeasy DSP FFPE Kit (cat. no. 73604), see the *RNeasy DSP FFPE Kit Handbook*.

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


The *therascreen* FGFR RGQ RT-PCR Kit is intended for in vitro diagnostic use.

For prescription use only.

For use with the Rotor-Gene Q MDx (US) instrument.

For use with the RNeasy DSP FFPE Kit.

The overall run control failure rate (PC and NTC) observed when using the *therascreen* FGFR RGQ RT-PCR Kit is approximately 5.2% (1% for the PC and 4.2% for NTC). In the event that an invalid run result is obtained, sample testing should be repeated as described in the Troubleshooting Guide, page 48, using available sample RNA. If insufficient RNA is available for retest from the original FFPE extraction, re-extraction from FFPE material is required.

<p>CAUTION</p> 	<p>DO NOT add bleach or acidic solutions directly to the sample preparation waste</p>
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The following hazard and precautionary statements apply to components of the *therascreen* 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

Always pay attention to the following.

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.

- This test is for use with clinical FFPE UC specimens only.
- 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 *therascreen* FGFR RGQ RT-PCR Kit are optimally diluted. Do not dilute reagents further as this may result in a loss of performance.
- Do not use reaction volumes (reaction mix plus sample) of less than 25 µl.
- The reagents supplied in the *therascreen* FGFR RGQ RT-PCR Kit are intended to be used only with other reagents from the same kit lot. Do not substitute any supplied reagents with the same reagents from a different *therascreen* FGFR RGQ RT-PCR Kit production lot, as this may affect performance.
- Refer to the Rotor-Gene Q MDx (US) 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.
 - Use individual, dedicated pipettes for setting up reaction mixes and adding templates.
 - Do not open the Rotor-Gene Q MDx (US) instrument until the run is finished.
 - Do not open Rotor-Gene Q tubes after the real-time PCR run is finished.
 - Caution must be used to ensure correct sample testing with emphasis placed upon avoiding incorrect sample entry, loading errors and pipetting errors.
 - Make sure the samples are handled in a systematic way and clearly labeled to ensure correct identification at all times, thereby maintaining traceability.

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 *therascreen* FGFR RGQ RT-PCR Kit is shipped on dry ice and must be frozen on arrival. If any component of the *therascreen* 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, handbooks, 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 *therascreen* 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 removed from the kit box and immediately stored at -90 to -65°C until the stated expiration date.

For storage information for the RNeasy DSP FFPE Kit (cat. no. 73604), see the *RNeasy DSP FFPE Handbook*.

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. Do not exceed a maximum of 5 freeze-thaw cycles.

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

Specimen Handling and Storage

FFPE specimens

The *therascreen* FGFR RGQ RT-PCR Kit is for use with RNA samples extracted from FFPE urothelial cancer (UC) specimens using the RNeasy DSP FFPE Kit.

RNA samples

Once extracted, RNA samples should either be tested immediately with the *therascreen* FGFR RGQ RT-PCR Kit or stored at -90 to -65°C . Do not exceed a maximum of 5 freeze-thaw cycles.

To prepare tissue samples for RNA extraction:

- Using a microtome, cut 4–5 μm serial sections from the FFPE sample block and mount them on glass slides.
- A trained individual (e.g., a pathologist) must 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 FFPE input required 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).

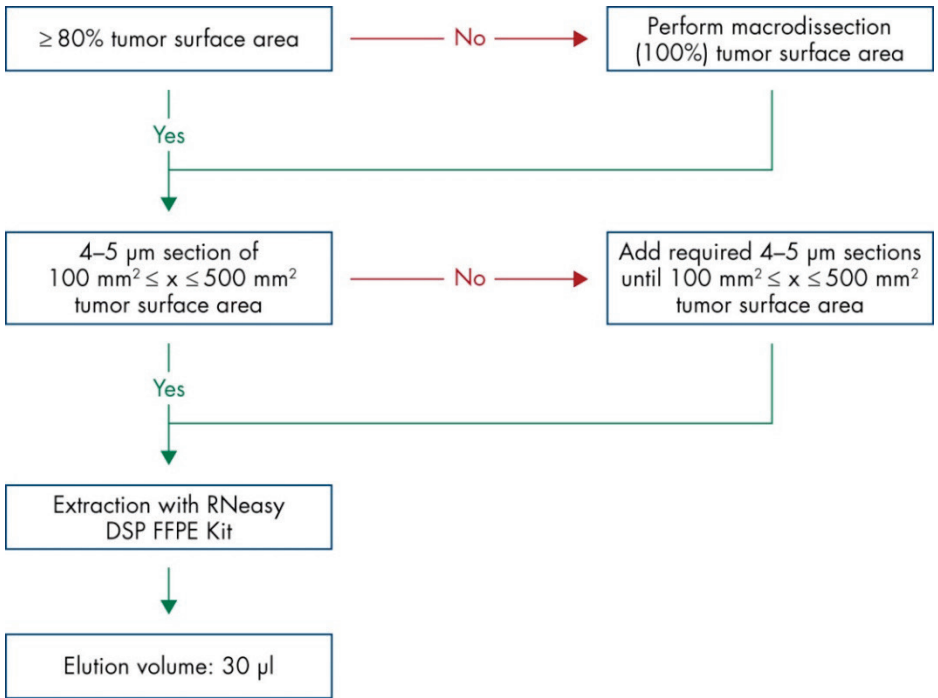


Figure 3. Clinical FFPE UC sample purification workflow to be used with the *therascreen* FGFR RGQ RT-PCR System.

- Macrodissection should be 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 must be used to achieve the minimum sample requirements.

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

Procedure

RNA extraction and preparation

RNA should be purified using the RNeasy DSP FFPE Kit (cat. no. 73604).

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

Note: Performance of the *therascreen* FGFR RGQ RT-PCR Kit has only been validated in combination with the RNeasy DSP FFPE Kit (cat. no. 73604). Do not use any other RNA purification product.

Important points before starting

- If using the RNeasy DSP FFPE Kit for the first time, read “Important Notes” in the *RNeasy DSP FFPE Kit Handbook*.
- If working with RNA for the first time, read “Appendix: General Remarks on Handling RNA” in the *RNeasy DSP 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* for safety information.
- Unless otherwise indicated, perform all steps of the procedure 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*.

- 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

- Follow the RNA purification procedure as described in the *RNeasy DSP FFPE Kit Handbook*. In the protocol “Purification of Total RNA from FFPE Tissue Sections”, ensure the volumes used for processing >3–4 sections (marked with ●) are followed.
- Elute RNA in 30 µl RNase-free water provided with the RNeasy DSP FFPE Kit.
- Aliquot 3 µl of eluted RNA for quantification.
- Store RNA eluates at –90 to –65°C.

Quantification and normalization of RNA

Procedure

- Blank the spectrophotometer using the RNase-free water supplied with the RNeasy DSP FFPE Kit that was used to elute RNA.
- RNA quantity is determined by measuring optical density at 260 nm.
- Total amount of RNA purified = concentration x volume of sample in µl.
- If the RNA concentration is below 16.67 ng/µl, the sample must not be processed further. A fresh RNA extraction from a new FFPE specimen should be used for further analysis.
- RNA must be diluted to 16.67 ng/µl using the Water for Sample Dil. provided in the *therascreen* FGFR RGQ RT-PCR Kit.
- 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 *therascreen* 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 *therascreen* FGFR RGQ RT-PCR Kit PCR reaction.

Important points before starting

- Set up all reactions at 0–8°C to minimize the risk of RNA degradation.
- RNase inhibitor and dNTPs are already included in the kit components. Do not add additional RNase inhibitor and dNTPs.
- RT Primer Mix (supplied) should be used. 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 must be stopped by incubation at 95°C for 3 minutes to deactivate 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 *therascreen* 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 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 three 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 enough 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: Prepare sufficient master mix for two 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 μ l
RT Buffer 2	2.5 μ l
RT Primer Mix	1.25 μ l
Reverse Transcriptase†	1.25 μ l
Total volume of master mix	10 μl

* Includes Mg^{2+} and dNTPs.

† Also contains RNase inhibitor.

8. Pulse vortex the RT reaction mix three 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 μ l of RT reaction mix into each 0.2 ml tube.
13. Pulse vortex the Water for NTC, PC Diluent, and PC provided with the *therascreen* FGFR RGQ RT-PCR Kit and normalized RNA samples three 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, set the pipette to 15 μ l, 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 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 μ l
Test sample	Sample	15 μ l
Positive control	Positive control (PC)	5 μ l
	PC Diluent	10 μ l

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. For short-term storage, samples can be stored at 2–8°C for a maximum of 5 days, or at –30 to –15°C for a maximum of 30 days.

Real-time PCR on the Rotor-Gene Q MDx (US) instrument

The *therascreen* FGFR RGQ RT-PCR Kit provides accurate real-time PCR detection of the specified FGFR alterations in a multiplex format. The *therascreen* 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.

Important point before starting

- The PCR must begin with an initial incubation step of 15 minutes at 95°C to activate the HotStarTaq DNA Polymerase.

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 *therascreen* 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 three 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	9
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	Tube 65 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	Tube 66 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	Tube 67 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	Tube 68 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	Tube 69 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	Tube 70 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	Tube 71 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	Tube 72 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.

- Vortex the cDNA samples for 3 seconds, then centrifuge briefly to collect the drops from the lids and sides of the tubes.
- 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 *therascreen* 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 *therascreen* FGFR FFPE MDx Assay Profile version 1.0.3 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 MDx (US) instrument is maintained.
12. Place the 72-well rotor into the Rotor-Gene Q MDx (US) instrument. Ensure that the locking ring (supplied with the Rotor-Gene Q MDx (US) 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”, page 32.

Note: The Rotor-Gene Q MDx (US) instrument cycling conditions for use with the *therascreen* 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 MDx (US) instrument. 
2. The "Setup" environment appears by default. Click **New manual work list** to create a new work list (Figure 6, next page).

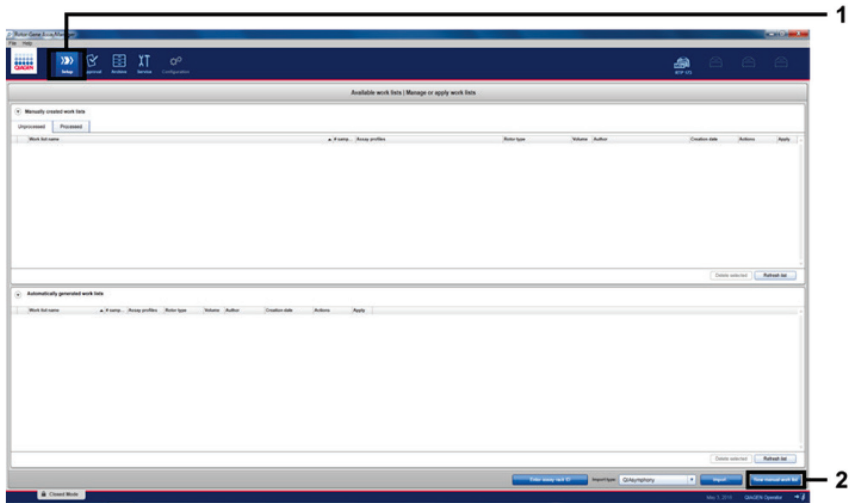


Figure 6. Setting up new manual work list. 1 = Select “Setup” environment, 2 = “New manual work list”.

3. Select the “Assays” tab on the left-hand side of the main window. Select **therascreen FGFR FFPE MDx Assay Profile** from the list of available assay profiles and click the blue arrow to select the assay profile. If the assay profile name is truncated, move the mouse pointer over the assay profile to see the full name (Figure 7).

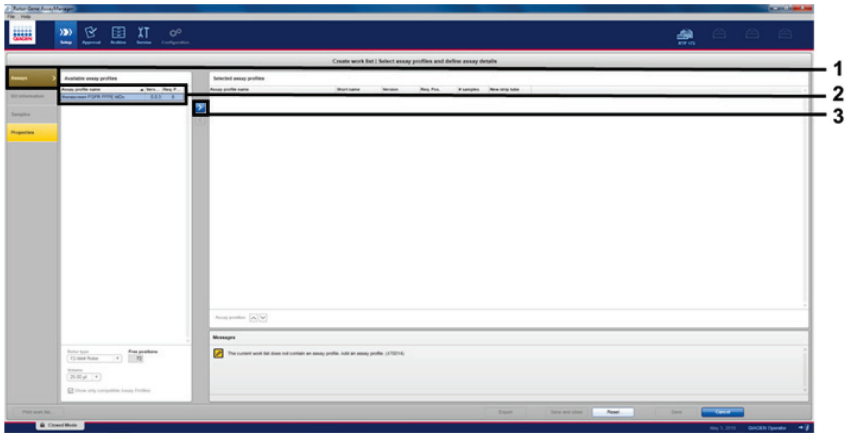


Figure 7. Setting up new manual work list; choosing assay profile name. 1 = “Assays” tab, 2 = Select “*therascreen FGFR FFPE MDx*” from the available assay profiles, 3 = Click on the arrow.

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

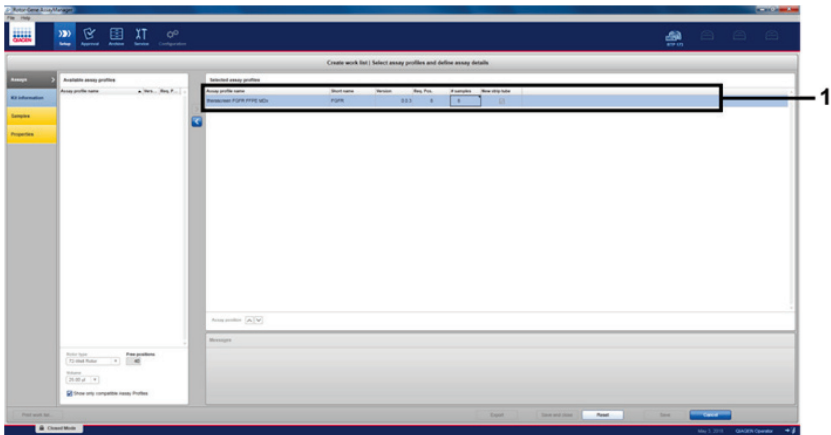


Figure 8. Create work list main window. 1 = Add the number of samples in the “Assay profile name”.

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

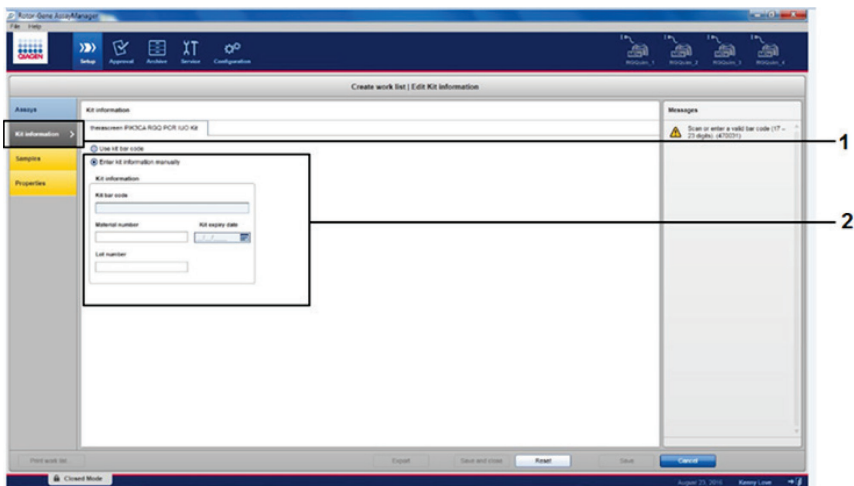


Figure 9. Create work list main window. 1 = “Kit information” tab, 2 = Enter the kit information.

6. Click the “Samples” tab to enter sample information. Enter the sample names manually (Figure 10, next page).

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

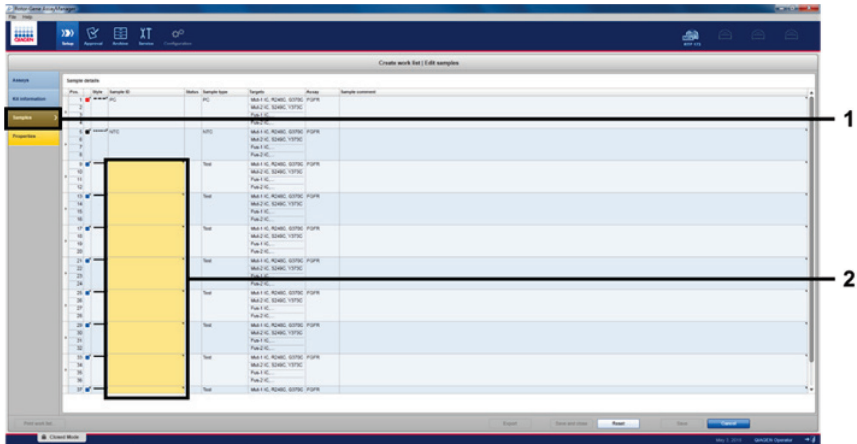


Figure 10. Create work list main window. 1 = “Samples” tab, 2 = Enter sample names.

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** checkboxes have been activated. Then, click **Apply** in the bottom right-hand corner to apply the work list (Figure 11, next page).

8. Enter the experiment name into the **Experiment name** field. Select a cyclor from the list of available cyclors 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 MDx (US) icon at the top right of the screen to indicate that the run has started (Figure 12).

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

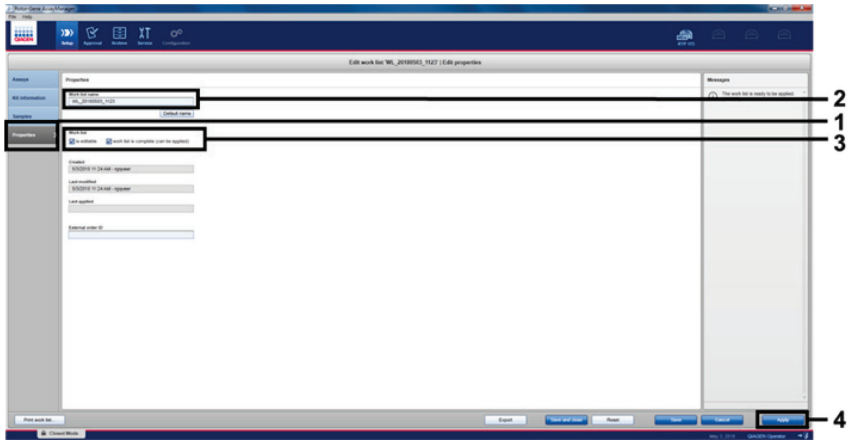


Figure 11. Create work list main window. 1 = "Properties" tab, 2 = Enter work list name, 3 = Select "is editable" and the "work list is complete", 4 = "Apply".

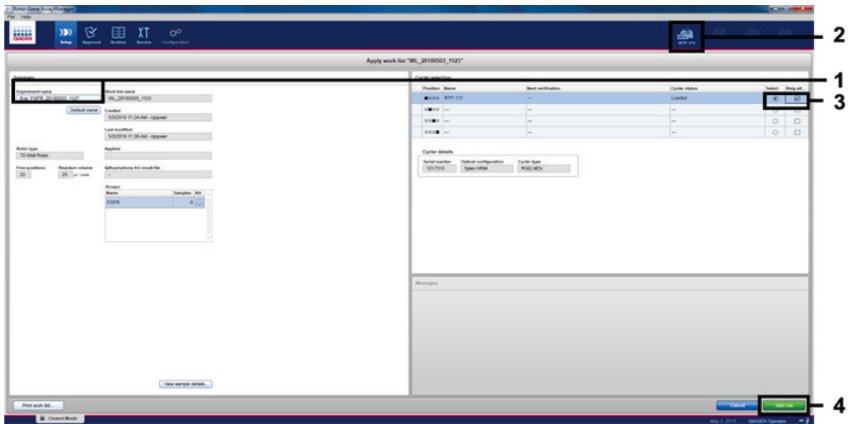


Figure 12. Applying work list and run start. 1 = Enter experiment name, 2 = Select the instrument, 3 = Ensure that "Ring attached" is selected, 4 = Start run.

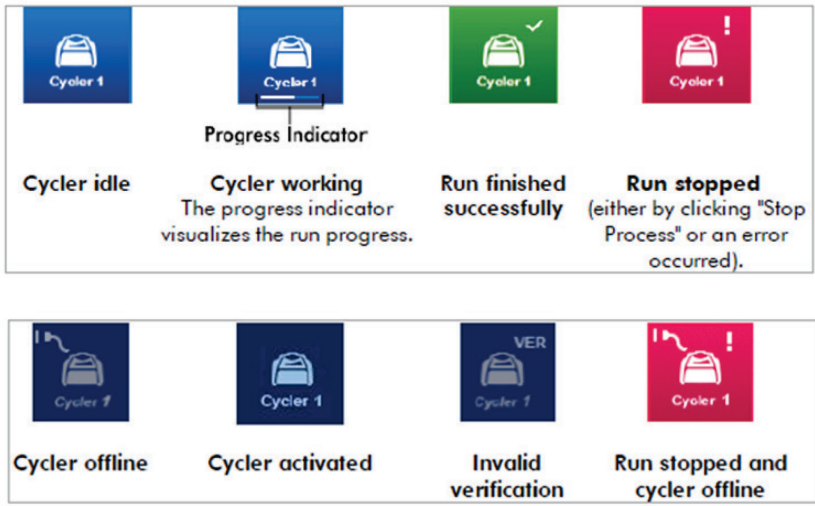


Figure 13. Rotor-Gene Q MDx (US) Cyclus icons that may be displayed.

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

Click **Release and go to approval** to enter the “Approval” environment and release the Rotor-Gene Q MDx (US) 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).

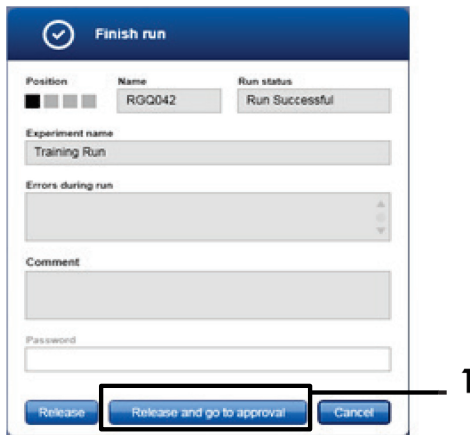


Figure 15. “Finish Run” pop up window. 1 = “Release and go to approval”.

10. Select the experiment in the “Assay selection” part of the Approval environment.

11. “Raw data”, “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, next page).

Note: The “*therascreen* FGFR FFPE MDx” assay profile file associated with the *therascreen* FGFR RGQ RT-PCR Kit contains all the software instructions required for automatic sample analysis and result interpretation. Following PCR cycling, the Rotor-Gene AssayManager software assesses the validity of the run controls and samples automatically. If the Positive Control and No Template Control data are within the preset acceptable range for the test, the run controls will report “Valid”. If both run controls are valid the samples will be analyzed on an individual basis; if the internal control data are within the pre-set acceptable range for the test, the sample will report “Valid”, if outside the pre-set acceptable range for the test the sample will report “Invalid”. If the external controls are outside of the pre-set acceptable range for the test, an “Invalid” overall sample result will be reported. If either of the run controls fail, the entire run will be invalidated. All samples will then be flagged as “ASSAY_INVALID”. If this occurs, see “Analysis” on page 43 for instructions regarding how to proceed.

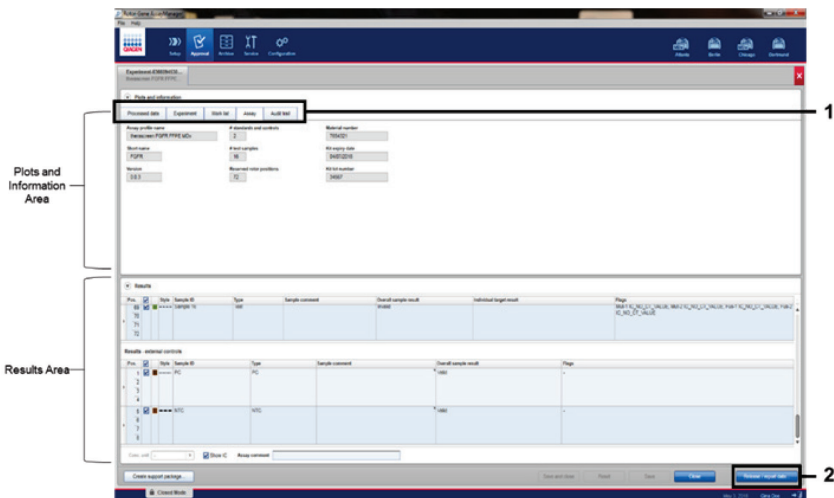


Figure 16. Example of assay results main windows. 1 = “Experiment” tab in the “Plots and information”. 2 = “Release/report data”.

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. 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.

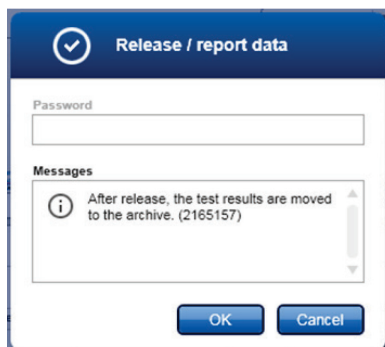


Figure 17. The “Release/report data” dialog box.

14. To view an experiment stored in the experiment archive, click the “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).
15. 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 MDx Plug-in User Manual*.

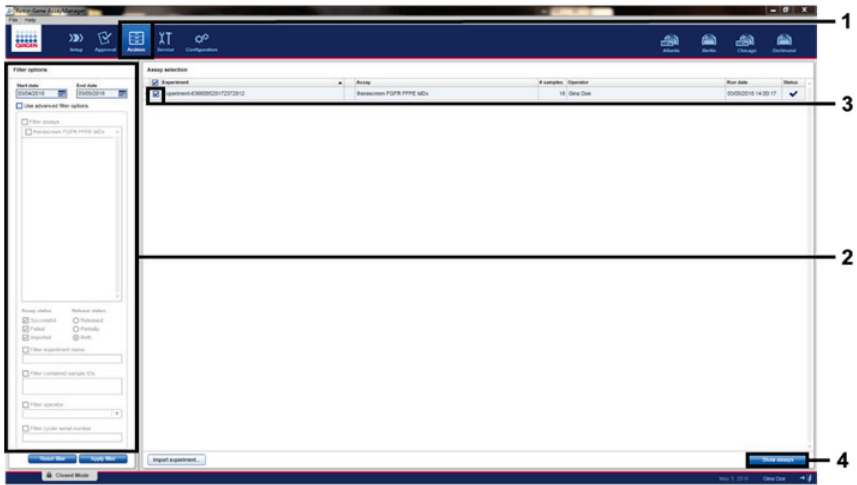


Figure 18. The “Experiment Archive” main window. 1 = “Archive” environment selection, 2 = Search options, 3 = Experiment name selection, 4 = “Show assays” control.

Analysis

All data analysis steps are performed automatically, with no requirement for manual interpretation. Rotor-Gene AssayManager automatically checks run validity and sample validity criteria and will not report mutation status results in the event of an invalid run or sample. The analytical result is determined by combining all relevant PCR data according to core analysis algorithms defined in the *therascreen* FGFR FFPE MDx assay profile.

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. This run control is valid if the C_T value of each PC is within the relevant PC specifications.
- The NTC reaction is checked for the absence of specific amplification within the no template control assays and the IC assays. This run control is valid if no C_T value is observed or if the C_T value is above the NTC specification.

If any of these run controls do not conform to specifications, the run will be invalidated, and the analysis will need to be performed again from the reverse transcription step.

If all run control analyses conform to specifications, analysis of the sample reactions is performed. Based on predetermined C_T values, the FGFR gene alteration status of each cDNA sample is qualitatively determined and reported.

The following results may be assigned to an individual sample:

- FGFR Alteration Detected ("Valid")
- No Alteration Detected ("Valid")
- INVALID: If one or more flags are assigned to the sample during analysis by Rotor-Gene AssayManager v2.1 that are defined to set the FGFR alteration result to "INVALID"

Note: A tumor may contain more than one FGFR alteration. In such instances, more than one FGFR alteration will be reported.

Note: The report generated at the end of the run shows the results obtained from the experimental samples and run controls, with invalidating flags displayed in front of any invalid results. If an error occurs during the RGQ 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 MDx Plug-in are listed in the *Rotor-Gene AssayManager v2.1 Gamma MDx Plug-in User Manual*. Additional flags which are specific to “*therascreen_FGFR_FFPE_MDx*” Assay Profile are shown in Table 6.

Table 6. Example software flags which may be displayed

Software Flags	When this flag is shown	Action to be taken
PC_ABOVE_ACCEPTED_RANGE	PC above the specification range	Invalid run. Test to be repeated from the extracted RNA if available, or re-extracted if it is not.
PC_BELOW_ACCEPTED_RANGE	PC below the specification range	Invalid run. Test to be repeated from the extracted RNA.
PC_NO_CT_VALUE	No C _T value for PC in PC tubes	Invalid run. Test to be repeated from the extracted RNA.
NTC_UNEXPECTED_CT_VALUE	C _T value detected in NTC	Invalid run. Test to be repeated from the extracted RNA.
(Alteration) IC_ABOVE_ACCEPTED_RANGE	Sample above specification range	Invalid sample. Sample to be re-extracted.
(Alteration) IC_BELOW_ACCEPTED_RANGE	Sample below specification range	Invalid sample. Sample to be re-extracted.
(Alteration) IC_NO_CT_VALUE	No C _T value for sample	Invalid sample. Test can be repeated from the extracted RNA or sample to be re-extracted.
(Alteration) UNEXPECTED_EARLY_CT	Amplification curve crossed threshold early in cycling	Anomalous sample result. Repeat test from the extracted RNA.

Note: If repeated invalid runs are obtained, contact QIAGEN Technical Services.

Limitations of the Procedure

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

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

The *therascreen* FGFR RGQ RT-PCR kit is also designed to identify FGFR2 fusions FGFR2-BICC1 and FGFR2-CASP7, and FGFR3 fusion FGFR3-BAIAP2L1 because these FGFR fusion patients were eligible for the trial. However, the test is not clinically validated to detect these three fusions due to a lack of required clinical specimens. Drug safety and efficacy has not been established for cases of UC harboring these fusions and no claims are made for the use of the *therascreen* FGFR RGQ RT-PCR kit as an aid in the selection of such patients for treatment with BALVERSA (erdafitinib).

Samples with results reported as “No FGFR Alteration Detected” may harbor FGFR alterations that are not detected by the *therascreen* FGFR RGQ RT-PCR Kit.

Detection of FGFR alterations is dependent on sample integrity and the amount of amplifiable cDNA which 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, the testing procedure should be repeated for this sample.

The product is to be used only by personnel specially instructed and trained in in vitro diagnostics procedures and operation of the Rotor-Gene Q MDx (US) instrument.

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

Strict compliance with the *therascreen 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 with automated FGFR alteration status calling, together with the Gamma MDx Plug-in version 1.0.0 and *therascreen FGFR FFPE MDx Assay Profile* version 1.0.3.

The *therascreen FGFR RGQ RT-PCR Kit* does not demonstrate any detectable cross-reactivity (resulting in a report of "Alteration Detected") between the FGFR gene alteration assays it includes.

The *therascreen FGFR RGQ RT-PCR Kit* provides a qualitative test result, generating either a positive or negative result call for each FGFR alteration.

The *therascreen 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 *therascreen 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 invalid result

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

Repeat the test from the RT step using sample RNA if available. If sample RNA is not available, repeat the test 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.

PC sample shows invalid 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, if necessary.

b) The storage conditions for one or more kit components did not comply with the instructions given in "Reagent Storage and Handling" (page 21)

Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.

c) The *therascreen* 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.

IC shows invalid result

IC is outside preset acceptable range; RNA sample quality is unsuitable for testing with the *therascreen* FGFR RGQ RT-PCR Kit

Repeat RNA extraction if sufficient FFPE tissue is available.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System (ISO 13485), each lot of the *therascreen* FGFR RGQ RT-PCR Kit is tested against predetermined specifications to ensure consistent product quality.

Performance Characteristics

Limit of blank (LoB)

The limit of blank (LoB) is defined in CLSI guideline EP17-A2 as “the highest result that can reasonably be expected from a blank sample (i.e., a sample with concentration at or near zero) for a given error probability α ”. For the *therascreen* FGFR RGQ RT-PCR Kit, this is the data point that corresponds to the upper 95th percentile in FGFR alteration-negative samples. The LoB was determined by measuring the levels of breakthrough for each of the nine FGFR alteration assays of the *therascreen* 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 *therascreen* FGFR RGQ RT-PCR Kit lots and three instruments. The LoB for each assay within the *therascreen* FGFR RGQ RT-PCR Kit is shown in Table 7.

Table 7. Summary of LoB results

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

* FGFR2 fusions FGFR2-BICC1 and FGFR2-CASP7 and FGFR3 fusion FGFR3-BAIAP2L1 have not been analytically validated by the *therascreen* FGFR RGQ RT-PCR Kit with clinical specimens.

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., FGFR alteration detected) and C_T values generated above the cutoff are classified as FGFR alteration-negative (i.e., no alteration 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 FGFR alteration detected classification. The cutoff for each assay within the *therascreen* FGFR RGQ RT-PCR Kit is shown in Table 8.

Table 8. Summary of assay cutoff results

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

* FGFR2 fusions FGFR2-BICC1 and FGFR2-CASP7 and FGFR3 fusion FGFR3-BAIAP2L1 have not been analytically validated by the *therascreen* FGFR RGQ RT-PCR Kit with clinical specimens.

Assay cross-reactivity and analytical specificity

Assay cross-reactivity

Assay cross-reactivity is defined as nonspecific amplification of an FGFR alteration by the *therascreen* 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 *therascreen* 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 *therascreen* FGFR RGQ RT-PCR Kit.

Analytical specificity

The level of potential cross-reactivity between primers, probes, and blockers used within the *therascreen* 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 *therascreen* 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 *therascreen* 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 *therascreen* 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 *therascreen* 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 *therascreen* FGFR RGQ RT-PCR Kit lots tested (Table 9).

Table 9. Summary of LoD results

FGFR assay	LoD (copies/μl)
p.R248C (c.742C>T)	75.80
p.S249C (c.746C>G)	289.82
p.G370C (c.1108G>T)	141.57
p.Y373C (c.1118A>G)	274.71
FGFR3-TACC3v1	25.26
FGFR3-TACC3v3	45.75
FGFR3-BAIAP2L1 *	9.07
FGFR2-BICC1 *	14.34
FGFR2-CASP7*	27.18

* FGFR2 fusions FGFR2-BICC1 and FGFR2-CASP7 and FGFR3 fusion FGFR3-BAIAP2L1 have not been analytically validated by the *therascreen* FGFR RGQ RT-PCR Kit with clinical specimens.

The LoDs of the 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 assays were verified using FGFR alteration-positive clinical UC samples.

Repeatability and reproducibility

The repeatability (within laboratory) of the *therascreen* FGFR RGQ RT-PCR Kit was assessed by testing contrived samples at 3x LoD, representing the nine alterations detected by the *therascreen* 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 10).

Table 10. 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.S249C (c.746C>G)	60/60	100%	94.04%	100.00%
p.G370C (c.1108G>T)	60/60	100%	94.04%	100.00%
p.Y373C (c.1118A>G)	60/60	100%	94.04%	100.00%
FGFR3-TACC3v1 *	59/60	98.3%	91.06%	99.96%
FGFR3-TACC3v3	59/60	98.3%	91.06%	99.96%
FGFR3-BAIAP2L1	60/60	100%	100.00%	94.04%
FGFR2-BICC1	60/60	100%	100.00%	94.04%
FGFR2-CASP7	60/60	100%	100.00%	94.04%
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 1xLoD 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 (1 internal QIAGEN site in the United Kingdom and two additional external sites in the USA). The contrived samples for all FGFR alterations at 3xLoD and wild-type samples were tested by three operators (per site) over five days using three Rotor-Gene Q MDx (US) 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 1xLoD clinical samples were tested at each of the three sites using the *therascreen* FGFR RGQ RT-PCR Kit (2 biological replicates x 2 *therascreen* FGFR RGQ RT-PCR Kit lots x 2 operator x 3 days = 24 replicates at each site). These total replicates were tested across three Rotor-Gene Q MDx (US) instruments at each site and two of the three *therascreen* FGFR RGQ RT-PCR Kit lots were used alternatively in each site (Table 11).

Table 11. 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	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	72/72	100.00%	95.01%	100.00%
p.G370C (c.1108G>T)	3x LoD	Contrived	120/120	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	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	72/72	100.00%	95.01%	100.00%
p.Y373C (c.1118A>G)	3x LoD	Contrived	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	71/72	98.61%	92.50%	99.96%
FGFR3-TACC3v3	3x LoD	Contrived	119/120	99.17%	95.44%	99.98%
	1x LoD	Clinical	71/72	98.61%	92.50%	99.96%
FGFR3-TACC3v1	1x LoD	Contrived	119/120	99.17%	95.44%	99.98%
	1/3x LoD	Clinical	63/72	87.50%	77.59%	94.12%
FGFR3-BAIAP2L1	3x LoD	Contrived	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	NA*	NA*	NA*	NA*
FGFR2-BICC1	3x LoD	Contrived	120/120	100.00%	96.97%	100.00%
	1x LoD	Clinical	NA*	NA*	NA*	NA*
FGFR2-CASP7	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%

* Indicates that clinical UC FFPE samples could not be sourced. 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., FGFR alteration 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 *therascreen* 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 *therascreen* 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 *therascreen* FGFR RGQ RT-PCR Kit. The FGFR System utilizes two separate kits: the RNeasy DSP FFPE Kit for isolation of RNA from clinical UC FFPE specimens and the *therascreen* 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 *therascreen* 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 *therascreen* 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-CASP7 and FGFR2-BICC1 alteration were produced at the 3xLoD level and tested using the three *therascreen* FGFR RGQ RT-PCR Kit lots. All samples were normalized and diluted to the 3xLoD 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 *therascreen* 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 *therascreen* 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 *therascreen* 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 *therascreen* 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.

Clinical performance

In FGFR-altered cases of UC, treatment with the pan-FGFR TKI drug BALVERSA (erdafitinib) has an overall response rate in 32.2% of patients by Blinded Independent Review Committee (BIRC) (5).

The *therascreen* FGFR RGQ RT-PCR Kit is intended to be used as a companion diagnostic test as an aid in identifying patients with cases of UC that harbor specific FGFR gene alterations and are therefore eligible for treatment with BALVERSA (erdafitinib).

Correlation to an orthogonal method

To demonstrate the accuracy of the *therascreen* FGFR RGQ RT-PCR Kit (relative to RT droplet digital PCR (RT-ddPCR)), an accuracy study was conducted with specimens from the 42756493-BLC2001 clinical trial, supplemented with specimens obtained from the same intended use (intent to diagnose) population. The *therascreen* FGFR RGQ RT-PCR Kit and ddPCR testing for FGFR alterations was performed on the same samples extracted from 307 specimens (271 clinical trial specimens and 36 procured specimens). Table 12 shows the frequency of results in agreement between the *therascreen* FGFR RGQ RT-PCR Kit and the orthogonal method.

Samples with valid results for both *therascreen* FGFR RGQ RT-PCR Kit and ddPCR (n = 306) were analyzed to assess the Positive Percent Agreement (PPA), Negative Percent Agreement (NPA) and Overall Percent Agreement (OPA), based on agreement between the two methods for overall FGFR alteration status (FGFR alteration detected or No alteration detected). These percentages, together with the corresponding two-sided 95% confidence intervals (CI) are summarized in Table 13. Table 14 shows the PPA of the target with ddPCR as the orthogonal method.

Table 12. Two-by-two frequency table (excluding invalid results) of the *therascreen* FGFR RGQ RT-PCR Kit vs. ddPCR call

<i>therascreen</i> FGFR RGQ RT-PCR Kit	Frequency	ddPCR		Total (percent)
		Negative	Positive	
Negative	197	1	198 (64.71)	
Positive	5	103	108 (35.29)	
Total (percent)	202 (66.01)	104 (33.99)	306 (100.00)	

Table 13. *therascreen* FGFR RGQ RT-PCR Kit versus ddPCR (with ddPCR as orthogonal method)

Measure of agreement	Percent agreement % (N)	Two-sided 95% CI
Positive percent agreement (PPA)	99.04% (103/104)	94.76, 99.98
Negative percent agreement (NPA)	97.52% (197/202)	94.32, 99.19
Overall percent agreement (OPA)	98.04% (300/306)	95.78, 99.28

Table 14. PPA with ddPCR as orthogonal method, along with the two-sided 95% confidence intervals by alterations

Alteration	Percentage agreement % (N correct/N Total)	Two-sided 95% CI
p.R248C (c.742C>T)	93.33% (14/15)	68.05, 99.83
p.S249C (c.746C>G)	100.00% (56/56)	93.62, 100.00
p.G370C (c.1108G>T)	100.00% (2/2)	15.81, 100.00
p.Y373C (c.1118A>G)	100.00% (18/18)	81.47, 100.00
FGFR3-TACC3v1	100.00% (16/16)	79.41, 100.00
FGFR3-TACC3v3	100.00% (5/5)	47.82, 100.00
FGFR3-BAIAP2L1	100.00% (1/1)	2.50, 100.00

For the six overall FGFR alteration status discordant results, one sample gave no alteration detected result by the *therascreen* FGFR RGQ RT-PCR Kit and gave alteration detected results by ddPCR while five samples gave FGFR alteration detected results by the *therascreen* FGFR RGQ RT-PCR Kit and gave no alteration detected results by ddPCR.

Clinical outcome data

The 42756493-BLC2001 trial was a Phase 2 study to determine the efficacy and the safety of the selected dose (8 mg once-daily) of BALVERSA (erdafitinib) in subjects with metastatic or surgically unresectable UC with FGFR genomic alterations. Eligible patients were required to have specific alterations in the FGFR2 or FGFR3 genes, as prospectively determined by the clinical trial assay (CTA). Retrospective testing of specimens from patients screened for the 42756493-BLC2001 clinical trial was performed using the companion diagnostic *therascreen* FGFR RGQ RT-PCR Kit.

A bridging study was conducted to assess the concordance of the *therascreen* FGFR RGQ RT-PCR Kit with the CTA used to select patients for the 42756493-BLC2001 clinical trial. The bridging study comprised 287 patient samples.

Samples with valid results for both *therascreen* FGFR RGQ RT-PCR Kit and CTA (n = 279) were analyzed to assess the PPA, NPA, and OPA, based on agreement between the two methods for overall FGFR gene alteration status (FGFR alteration detected or FGFR alteration not detected). These percentages, together with the corresponding two-sided 95% CI are summarized in Table 15.

Table 15. *therascreen* FGFR RGQ RT-PCR Kit versus CTA (with CTA as reference method)

Measure of agreement	Percent agreement % (N)	Two-sided 95% CI
Positive percent agreement (PPA)	85.2% (69/81)	75.9, 91.3
Negative percent agreement (NPA)*	97.0% (192/198)	93.5, 98.6
Overall percent agreement (OPA)	93.5% (261/279)	90.0, 96.1

* Prior chemotherapy information was not collected for CTA-negative patients. Therefore, 198 CTA-negative subjects included both Chemo Relapsed/Refractory and Chemo Naïve patients.

For the 18 overall FGFR alteration status discordant results, 12 samples gave a “No alteration detected” result by the *therascreen* FGFR RGQ RT-PCR Kit but gave “FGFR alteration detected” results by the CTA, while six samples gave “FGFR alteration detected” results by the *therascreen* FGFR RGQ RT-PCR Kit but gave “No alteration detected” results by the CTA.

The primary objective of the 42756493-BLC2001 study was to evaluate the objective response rate of subjects who were FGFR alteration-positive with the *therascreen* FGFR RGQ RT-PCR Kit (FGFR Kit+) (objective response rate [ORR] = complete response [CR] + partial response [PR]) by RECIST criteria as assessed by blinded independent review committee (BIRC). The observed clinical benefit in the subset of patients that were FGFR alteration detected with the *therascreen* FGFR RGQ RT-PCR Kit (n = 69) was comparable to that observed in the full study population, as defined by the CTA (n = 87).

The ORR with the *therascreen* FGFR RGQ RT-PCR Kit+ /CTA+ alterations by BIRC was 33.3% (95% CI: 23.4% – 45.1%). As there was no ORR data for FGFR2-BICC1, FGFR2-CASP7, and FGFR3-BAIAP2L1 fusions, it is not possible to claim that clinical validity for these alterations has been demonstrated.

Given that the *therascreen* FGFR RGQ RT-PCR Kit was not used to select patients for the 42756493-BLC2001 clinical trial, additional efficacy analyses were conducted to consider patients who were not included in the trial because the result from testing their sample using the CTA was no FGFR alteration, but who may have been assigned a result of FGFR alteration detected had the sample been tested by the *therascreen* FGFR RGQ RT-PCR Kit (i.e., *therascreen* FGFR RGQ RT-PCR Kit+/CTA-), as well as patients who were enrolled in the trial but did not have valid retest results from the *therascreen* FGFR RGQ RT-PCR Kit (i.e., *therascreen* FGFR RGQ RT-PCR Kit unknown/CTA+). The weighted overall ORR in all FGFR Kit+ subjects in the Chemo-Relapsed/Refractory Population were 33.3% (95% CI: (21.7% – 45.1%), 32.3% (95% CI: 21.2% – 43.4%), 31.4% (95% CI: 20.7% – 42.1%), 30.4% (95% CI: 20.1% – 41.1%) and 29.4% (95% CI: 19.3% – 40.0%) when a range of ORR values for the positive discordant subjects (CDx+/CTA-), were assumed as 100%, 75%, 50%, 25%, and 0% of the observed ORR in the positive concordant subjects (CDx+/CTA+).














Refer to the therapeutic product label at Drugs@fda.

References

1. Ornitz, D.M. and Itoh, N. (2015) The Fibroblast Growth Factor signaling pathway. Wiley Interdiscip. Rev. Dev. Biol. **4**, 215.
2. Knowles, M.A. and Hurst, C.D. (2015) Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat. Rev. Cancer **15**, 25.
3. Rodriguez-Vida, A., Saggese, M., Hughes, S., et al. (2015) Complexity of FGFR signaling in metastatic urothelial cancer. J. Hematol. Oncol. **24**, 119.
4. 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.
5. BALVERSA (Erdafitinib) US Prescribing Information.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
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	Lot number
	Material number
	Components
	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
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Product	Contents	Cat. no.
<i>therascreen</i> 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	874721
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
Rotor-Gene Q MDx (US) and accessories		
Rotor-Gene Q MDx System (US)	Real-time PCR cycler with 6 channels, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002036
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

Product	Contents	Cat. no.
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

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Handbook Revision History

Document	Changes	Date
HB-2838-001	Initial release of Instructions for Use for kit Version 2	September 2021
HB-2838-002	Updated Assay Profile version from v1.0.2 to v1.0.3	October 2021
	Updated Table 6 to include new software flag and recommended actions	
	Minor typographical edits for clarity	

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