

September 2019

therascreen[®] PIK3CA RGQ PCR Kit Instructions for Use (Handbook)



24

Version 1



For in vitro diagnostic use

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

For use with QIAamp[®] DSP DNA FFPE Tissue Kit

For use with QIAamp[®] DSP Circulating Nucleic Acid Kit



REF

873111



QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden,
Germany

R2 MAT

1116336EN

Sample to Insight



Contents

Intended Use	5
Limitations of the Procedure	6
Summary and Explanation of the Test	8
Principle of the Procedure	10
Mutation reaction mixes	10
Platform and Software	15
Materials Provided	16
Kit contents	16
Materials Required but Not Provided	17
Warnings and Precautions	19
General precautions	20
Reagent Storage and Handling	22
Shipping conditions	22
Storage conditions	22
Stability	22
Specimen Storage and Handling	24
Specimen storage	26
Procedure	27
DNA extraction from FFPE specimens	27
DNA extraction from plasma specimens	29
Detection of <i>PIK3CA</i> mutations	30
Performing a <i>PIK3CA</i> mutation analysis run	36

Results	50
Analysis	50
Rotor-Gene AssayManager v2.1 <i>therascreen PIK3CA</i> Assay Profile flags	52
Performance Characteristics: Tissue Specimens.....	56
Analytical performance: Tissue specimens	56
Limit of blank (LoB): Tissue specimens.....	56
Limit of detection (LoD): Tissue specimens	57
Genomic DNA input range: Tissue specimens.....	58
ΔC_T cutoff values: Tissue specimens	59
Effect of DNA input on ΔC_T values (linearity): Tissue specimens	60
Assay specificity (cross-reactivity/specificity): Tissue specimens	61
Interference: Tissue specimens.....	62
Lot interchangeability: Tissue specimens	64
Specimen handling: Tissue specimens	64
Repeatability and reproducibility: Tissue specimens	65
Cross-contamination/analytical carryover: Tissue specimens.....	68
Accuracy: Comparison to the analytical reference method (tissue specimens)	69
Clinical performance: Tissue specimens.....	71
Performance Characteristics: Plasma Specimens	76
Analytical performance: Plasma specimens.....	76
Limit of blank (LoB): Plasma specimens	76
Limit of detection (LoD): Plasma specimens.....	77
Genomic DNA input range: Plasma specimens	78
ΔC_T cutoff values: Plasma specimens	79

Effect of DNA input on ΔC_T values (linearity): Plasma specimens	80
Assay specificity (cross-reactivity/specificity): Plasma specimens	80
Interference: Plasma specimens	81
Lot interchangeability: Plasma specimens	82
Specimen handling: Plasma specimens	82
Repeatability and reproducibility: Plasma specimens	83
Blood collection tube validation.....	87
Accuracy: Comparison to the analytical reference method (plasma specimens)	88
Clinical performance: Plasma specimens	89
Troubleshooting Guide	95
References	97
Contact Information	97
Symbols.....	98
Ordering Information	100
Document Revision History	102

Intended Use

The *therascreen PIK3CA RGQ PCR Kit* is a real-time qualitative PCR test for the detection of 11 mutations in the phosphatidylinositol 3-kinase catalytic subunit alpha (*PIK3CA*) gene (Exon 7: C420R; Exon 9: E542K, E545A, E545D [1635G>T only], E545G, E545K, Q546E, Q546R; and Exon 20: H1047L, H1047R, H1047Y) using genomic DNA (gDNA) extracted from formalin-fixed, paraffin-embedded (FFPE) breast tumor tissue or circulating tumor DNA (ctDNA) from plasma derived from K₂EDTA anticoagulated peripheral whole blood taken from patients with breast cancer.

The *therascreen PIK3CA RGQ PCR Kit* is intended for use as a companion diagnostic test, to aid clinicians in identification of breast cancer patients who may be eligible for treatment with PIQRAY® (alpelisib) based on a *PIK3CA* mutation detected result. Patients whose FFPE tissue or plasma specimen produces a positive *therascreen PIK3CA RGQ PCR Kit* test result for the presence of one or more *PIK3CA* mutations are eligible for treatment with PIQRAY (alpelisib). Patients whose plasma specimen produces a negative result using this test should be reflexed to testing of a FFPE tumor tissue specimen for the presence of *PIK3CA* mutations.

FFPE tumor specimens are processed using the QIAamp DSP DNA FFPE Tissue Kit for manual sample preparation. K₂EDTA anticoagulated whole peripheral venous blood plasma specimens are processed using the QIAamp DSP Circulating Nucleic Acid Kit for manual sample preparation. For both specimen types, the Rotor-Gene Q (RGQ) MDx 5plex HRM instrument is used for automated amplification and detection.

The *therascreen PIK3CA RGQ PCR Kit* is an in vitro diagnostic medical device.

The *therascreen PIK3CA RGQ PCR Kit* is to be used by trained personnel in a professional laboratory environment.

Limitations of the Procedure

- This Instructions for Use document must be completely read and understood before the *therascreen PIK3CA RGQ PCR Kit* is used.
- 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.
- Samples with results reported as "No Mutation Detected" may harbor *PIK3CA* mutations not detected by the *therascreen PIK3CA RGQ PCR Kit*.
- Analytical and clinical performance data relating to detection of the following *PIK3CA* mutations: E545A, E545D, Q546E, Q546R and H1047Y were established using contrived plasma specimens (cell-line DNA spiked into plasma) only, not using clinical specimens from the Intended Use population.
- Detection of mutations is dependent on sample integrity and the amount of amplifiable DNA present. The test procedure should be repeated if analysis of the DNA in the sample indicates that the quantity and/or quality is either not sufficient or the concentration is too high for mutation analysis.
- The *therascreen PIK3CA RGQ PCR Kit* is used in a PCR procedure. As with all PCR procedures, samples may become contaminated by external sources of DNA in the test environment and the DNA in the positive control. Use caution to avoid contamination of samples and kit reagents.
- If the sample contains less than the percentage of mutant alleles that is able to be detected by the *therascreen PIK3CA RGQ PCR Kit*, it will lead to a result of "No Mutation Detected".
- It is not known whether the *therascreen PIK3CA RGQ PCR Kit* shows cross-reactivity (leading to a result of "Mutation Detected") to additional *PIK3CA* mutations besides those listed as biomarkers detected by the kit.
- The *therascreen PIK3CA RGQ PCR Kit* is a qualitative test. The test will not provide quantitative measurements of the Mutant Allele Frequency (MAF) present in a sample.

- The impact on the performance of the *therascreen PIK3CA RGQ PCR Kit* is unknown if microbial contamination is introduced during assay procedures; 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.
- The *therascreen PIK3CA RGQ PCR Kit* is only for use with DNA extracted from FFPE breast cancer tissue or plasma specimens prepared from K₂EDTA anticoagulated whole peripheral venous blood taken from breast cancer patients.
- The *therascreen PIK3CA RGQ PCR Kit* is only for use with the QIAamp DSP DNA FFPE Tissue Kit (for tissue specimens) or the QIAamp DSP Circulating Nucleic Acid Kit (for plasma specimens).
- The *therascreen PIK3CA RGQ PCR Kit* is only for use when all reaction mixes are used.
- The product is to be used only by personnel specially instructed and trained in in vitro diagnostic procedures and operation of Rotor-Gene Q MDx 5plex HRM instruments.
- The product is intended for use only on a Rotor-Gene Q MDx 5plex HRM real-time PCR cycler. No other thermal cycler with real-time optical detection can be used with this product.
- Strict compliance with the *therascreen PIK3CA RGQ PCR Kit Instructions for Use (Handbook)* is required for optimal results. Dilution of the reagents is not recommended and will result in a loss of performance.
- This handbook is intended to be used with Rotor-Gene AssayManager software version 2.1 with automated mutation status calling.
- 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.

Summary and Explanation of the Test

The phosphatidylinositol 3-kinase (PI3K) signaling pathway regulates diverse cellular functions, including cell proliferation, survival, translational regulation of protein synthesis, glucose metabolism, cell migration, and angiogenesis (1). Activating somatic missense mutations of the *PIK3CA* (phosphatidylinositol 3-kinase catalytic subunit alpha) gene that increase the kinase activity of the PI3K α protein have been identified in tumor tissues and have been linked to cellular transformation in many different human cancers (2), including hormone receptor positive (HR+) breast cancer (3).

Breast cancer is the most common cancer diagnosed in women and the second leading cause of cancer-related death (4). In 2018, it was estimated that 266,120 women would be diagnosed with breast cancer in the United States (representing approximately 30% of all cancers in women) and that 40,920 deaths would be recorded (5). In Europe, 92,700 women were predicted to die from breast cancer in 2018 (6). Breast cancer in men is rare, with <1% of breast cancer diagnoses in male patients (4); however, treatment recommendations are the same for both sexes.

The *therascreen* PIK3CA RGQ PCR Kit is a real-time qualitative PCR in vitro diagnostic test, performed on the Rotor-Gene Q MDx 5plex HRM instrument. It uses allele refractory mutation system (ARMS) primers, hydrolysis probes and PCR clamp technologies to detect 11 mutations (Table 1) in exons 7, 9, and 20 of the *PIK3CA* oncogene against a background of wild-type (WT) DNA.

Table 1. *therascreen PIK3CA RGQ PCR Kit* assay targets

Exon	Mutation	COSMIC* ID	Base change
7	C420R	757	1258 T>C
9	E542K	760	1624 G>A
	E545A	12458	1634 A>C
	E545D	765	1635 G>T
	E545G	764	1634 A>G
	E545K	763	1633 G>A
	Q546E	6147	1636 C>G
	Q546R	12459	1637 A>G
20	H1047L	776	3140 A>T
	H1047R	775	3140 A>G
	H1047Y	774	3139 C>T

* COSMIC: Catalogue of somatic mutations in cancer: <https://cancer.sanger.ac.uk/cosmic>.

Principle of the Procedure

The *therascreen* PIK3CA RGQ PCR Kit is comprised of six separate PCR amplification reaction mixes:

- Five mutation-specific reactions targeting exons 7, 9, and 20 of the *PIK3CA* gene
- One control reaction targeting exon 15

The principal components of the kit are explained below.

Mutation reaction mixes

Mutated DNA is selectively amplified and detected by mutation-specific reaction mixes using mutation-specific ARMS primers, probes (hydrolysis probes and short highly specific probes), and PCR clamps. Mutation reactions are detected in the Green, Yellow, and Crimson Channels of the Rotor-Gene Q MDx 5plex HRM instrument.

ARMS

Allele-specific amplification is achieved by ARMS, which exploits the ability of *Taq* DNA polymerase to distinguish between a matched and a mismatched base at the 3' end of a PCR primer. When the primer is fully matched, the amplification proceeds with full efficiency. When the 3' base is mismatched, only low-level background amplification may occur. Therefore, a mutated sequence is selectively amplified even in samples where the majority of the DNA does not carry the mutation (Figure 1).

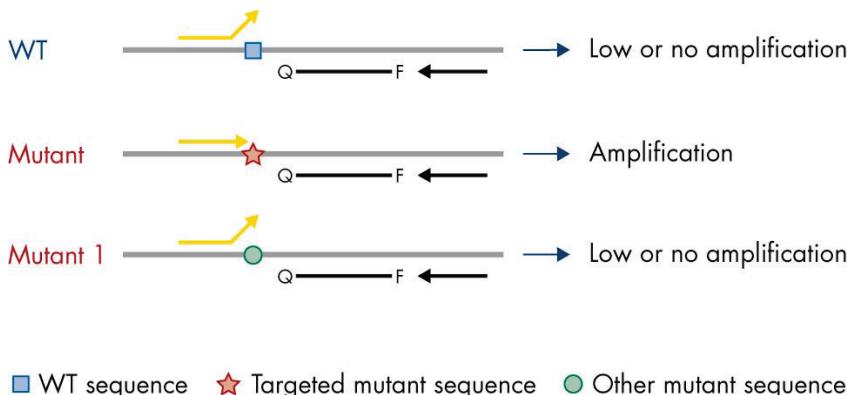


Figure 1. Identification of specific mutation by ARMS PCR. WT: Wild type. Q–F: Double-dye probe. ↗: Forward and reverse primers.

Hydrolysis probes

Hydrolysis probes anneal within a DNA region amplified by a specific set of primers. As the *Taq* polymerase extends the primer and synthesizes the nascent strand, the 5' to 3' exonuclease activity of the *Taq* polymerase degrades the probe, which leads to fluorophore release and fluorescence emittance.

The increase in fluorescence signal is detected only if the target sequence is complementary to the primers and probe and hence amplified during PCR (Figure 2).

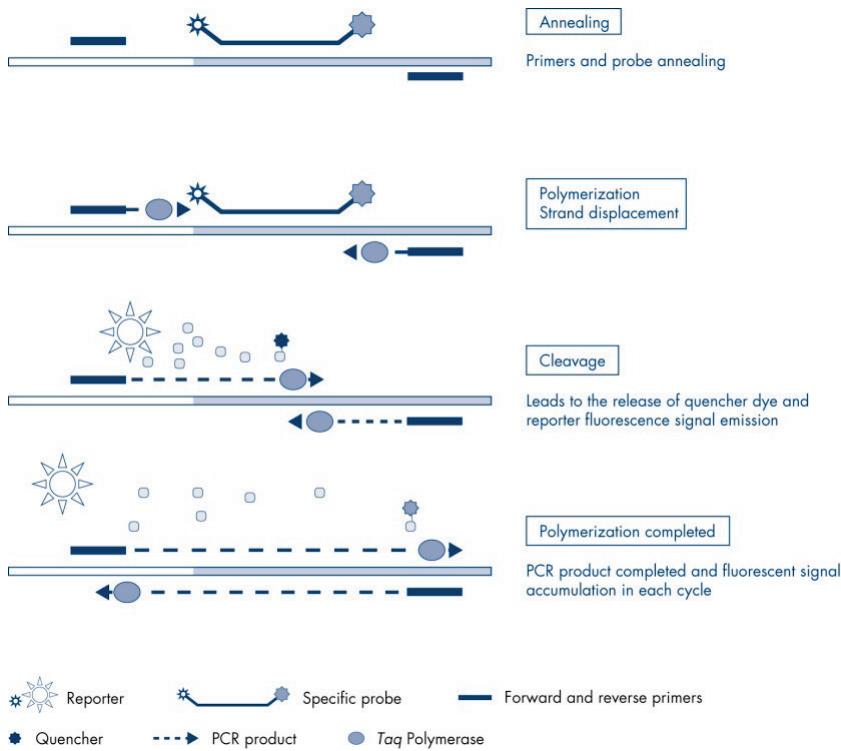


Figure 2. Reaction principle with hydrolysis probes.

PCR clamp

PCR clamps allow selective amplification of the mutant allele. PCR clamps that are perfectly matched to wild-type sequence bind to wild-type template and prevent amplification by interference with primer elongation. The 3' end of the PCR clamp is blocked with the addition of a phosphate group to prevent elongation of wild-type sequence (Figure 3).

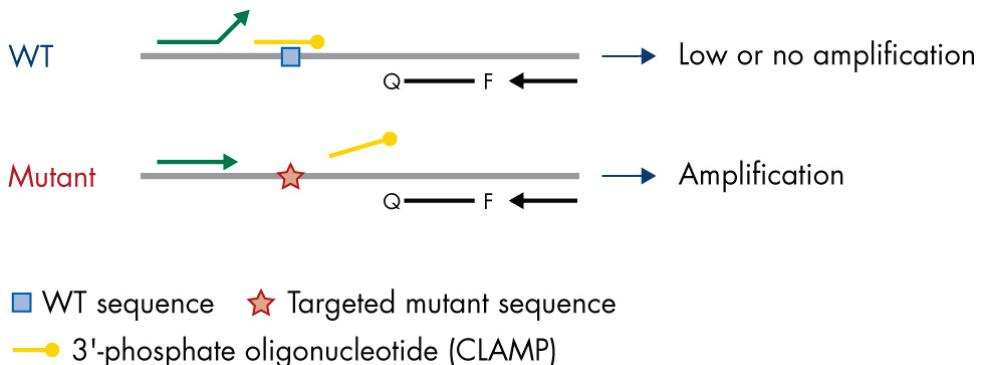


Figure 3. PCR clamp technology. WT: Wild type. Q—F: Double-dye probe. ←: Forward and reverse primers.

Control reaction

The Control Reaction Mix (Tube 1) contains a forward and reverse primer and labeled probe (detected in the Green Channel) to amplify a short sequence of exon 15 of the *PIK3CA* gene. The Control Reaction is used to determine if an appropriate level of amplifiable DNA is present in the sample and is a factor in the analytical calculations that determine mutation status.

Internal control

Each of the reaction mixes contains an Internal Control designed to detect failure of the reaction (e.g., due to the presence of inhibitors). The Internal Control employs a non-*PIK3CA* related oligonucleotide target sequence, unlabeled forward and reverse primers and a hydrolysis probe labeled with an orange fluorophore.

Positive control

The Positive Control (Tube PC) comprises a mixture of five plasmids representing each of the 11 mutations and the control. Detection of the mutations within acceptable ranges confirms the proper functioning of each of the reaction mixes in the kit.

Negative control

The No Template Control (Tube NTC) contains nuclease-free water to be used for the “No Template Control” (NTC) reaction. The NTC serves as a negative control and identifies potential contamination during assay setup.

Sample diluent

The Sample Diluent (Tube Dil.) contains nuclease-free water.

Platform and Software

The *therascreen PIK3CA RGQ PCR Kit* is specifically designed for use with the Rotor-Gene Q MDx instrument operating with a personal computer installed with:

- Rotor-Gene AssayManager® version 2.1
- Gamma Plug-in version 1.0.0
- *therascreen_PIK3CA_FFPE Assay Profile* version 1.0.1 for analysis of tissue specimens
- *therascreen_PIK3CA_Plasma Assay Profile* version 1.0.1 for analysis of plasma specimens

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

Refer to the *Rotor-Gene AssayManager v2.1 Core Application User Manual* and the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual* for further information concerning the software.

Run parameters

The Rotor-Gene Q MDx 5plex HRM instrument is programmed for different cycle parameters (or "runs") with the *therascreen PIK3CA Assay Profiles*. The Assay Profiles contain the PCR run parameters and calculate the results. PCR thermal cycling parameters for the assay are as follows:

- Hold at 95°C for 15 minutes to activate the *Taq DNA polymerase*.
- PCR for 45 cycles at 95°C for 30 seconds to denature, and 60°C for 1 minute to anneal and extend.

Materials Provided

Kit contents

<i>therascreen PIK3CA RGQ PCR Kit (24)</i>		
Catalog no.		873111
Number of reactions		24
Contents	Cap color	Volume
PIK3CA Reaction Mix 1	Red	750 µl
PIK3CA Reaction Mix 2	Purple	750 µl
PIK3CA Reaction Mix 3	Orange	750 µl
PIK3CA Reaction Mix 4	Yellow	750 µl
PIK3CA Reaction Mix 5	Green	750 µl
PIK3CA Reaction Mix 6	Blue	750 µl
Taq DNA Polymerase (<i>Taq</i>)	Mint	85 µl
PIK3CA Positive Control (PC)	Beige	250 µl
Water for No Template Control (NTC)	White	1.9 ml
Nuclease-free water for Dilution (Dil.)	White	1.9 ml
<i>therascreen PIK3CA RGQ PCR Kit Instructions for Use (Handbook)</i>		1

Materials Required but Not Provided

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

Reagents

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404, see "DNA extraction from FFPE specimens", page 27) or QIAamp DSP Circulating Nucleic Acid Kit (QIAGEN, cat. no. 61504, see "DNA extraction from plasma specimens", page 29)
- DNAZap™ PCR-degrading solutions
- Distel High Level Laboratory Disinfectant and isopropyl alcohol (IPA) wash

Consumables

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

Equipment

- Permanent marker
- Rotor-Gene Q MDx 5plex HRM Platform (cat. no. 9002032) or Rotor-Gene Q MDx 5plex HRM System (cat. no. 9002033)*†

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

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

- Rotor-Gene AssayManager v2.1, Gamma Plug-in and "therascreen_PIK3CA_FFPE" and/or "therascreen_PIK3CA_Plasma" Assay Profile
- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR master mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA
- Benchtop centrifuge* with rotor for 1.5 ml tubes
- Thermomixer*, heated orbital incubator*, heating block* or water bath* capable of incubation at 56°C, 70°C, and 90°C
- QIAvac 24 Plus vacuum manifold (cat. no. 19413)
- QIAvac Connecting System (cat. no. 19419)
- Vacuum Pump (cat. no. 84010) or equivalent pump capable of producing a vacuum of –800 to –900 mbar
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction set up (QIAGEN, cat. no. 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 (QIAGEN, cat. no. 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 (QIAGEN, cat. no. 9018903)
- Locking Ring 72-Well Rotor, for locking Strip Tubes and Caps, 0.1 ml in the 72-Well Rotor (QIAGEN, cat. no. 9018904)

Warnings and Precautions

For in vitro diagnostic use.

The *therascreen PIK3CA RGQ PCR Kit* is to be used by trained personnel in a professional laboratory environment.

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 use only with Rotor-Gene Q MDx 5plex HRM instrument.

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

Tissue specimens only: For use only with QIAamp DSP DNA FFPE Tissue Kit.

For safety information for the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404), see the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.

Plasma specimens only: For use only with QIAamp DSP Circulating Nucleic Acid Kit.

For safety information for the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504), see the *QIAamp DSP Circulating Nucleic Acid Kit Handbook*.

General precautions

- The test is for use with FFPE breast cancer tissue specimens or K₂EDTA plasma specimens from breast cancer patients.
- All chemicals and biological materials are potentially hazardous. FFPE specimen material and nucleic acids prepared from it are unlikely to pose an infection hazard, but all plasma specimens should be treated as potentially hazardous. Local institutional Health and Safety procedures must always be adhered to.
- Discard specimen, sample and assay waste according to your local safety procedures.
- Reagents for the *therascreen PIK3CA RGQ PCR Kit* are diluted optimally. Do not dilute reagents further as this may result in a loss of performance. Do not use reaction volumes (reaction mix plus sample) of less than 25 µl.
- All reagents supplied in the *therascreen PIK3CA RGQ PCR Kit* are intended to be used solely with the other reagents supplied in the same *therascreen PIK3CA RGQ PCR Kit*. Do not substitute the reagents in the *therascreen PIK3CA RGQ PCR Kit* or between *therascreen PIK3CA RGQ PCR Kits*, as this may affect performance.
- Only use the *Taq DNA Polymerase (Tube Taq)* that is provided in the *therascreen PIK3CA RGQ PCR Kit*. Do not substitute with *Taq DNA Polymerase* from other QIAGEN kits or with *Taq DNA Polymerase* from another supplier.
- Refer to the Rotor-Gene Q MDx 5plex HRM instrument user manual for additional warnings, precautions, and procedures.
- Do not use expired or incorrectly stored components.
- Use extreme caution to prevent contamination of the control and reaction mix reagents with the synthetic materials that are contained in the positive control reagent.
- Use extreme caution to prevent cross-contamination between samples. Cap the tubes promptly after addition of each sample.

- Thoroughly decontaminate the loading block before using it for preparation of assay master mixes. The use of DNAZap PCR-degrading solutions followed by Distel High Level Laboratory Disinfectant and IPA wash is recommended. The loading block must be dry before use.
- Use individual, dedicated pipettes for setting up reaction mixes and adding positive control reagents.
- Perform preparation and dispensing of reaction mixes in an area separate from the one used for the addition of the positive control.
- Fluorescently labeled molecules included in the reaction mix reagents are light sensitive. Protect control and reaction mix reagents from light to avoid photo bleaching.
- Do not open the Rotor-Gene Q MDx 5plex HRM instrument until the run has finished.
- Do not open Rotor-Gene Q tubes after the run has finished.
- Caution must be exercised to ensure correct sample testing to avoid incorrect sample entry, loading errors and pipetting errors.

Reagent Storage and Handling

Shipping conditions

The *therascreen* PIK3CA RGQ PCR Kit is shipped on dry ice and must be frozen on arrival. If any component of the *therascreen* PIK3CA RGQ PCR Kit is not frozen on arrival, if the outer packaging has been opened during transit or the shipment does not contain a packing note, Instructions for Use or the reagents, please contact QIAGEN Technical Services or local distributors (visit www.qiagen.com).

Storage conditions

The *therascreen* PIK3CA RGQ PCR Kit should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer and protected from light.

When stored under the specified storage conditions, the *therascreen* PIK3CA RGQ PCR Kit is stable until the stated expiration date.

Stability

Once opened, reagents can be stored in their original packaging at –30 to –15°C for 12 months or until the stated expiry date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of five freeze–thaw cycles.

The reagents must be thawed at room temperature for a minimum of 1 hour (and up to a maximum of 4.5 hours) prior to use. Once the reagents are ready to use, the PCR reactions can be set up. The Rotor-Gene Q tubes, containing the master mixes and the sample DNA, should be loaded onto the Rotor-Gene Q MDx immediately. The total time from start of PCR setup to the start of the run should not exceed **7.5 hours** if carried out at ambient temperature.

Note: This time includes both the PCR setup and storage.

Note: Fluorescently labeled molecules included in the reaction mix reagents are light sensitive. Protect control and reaction mix reagents from light to avoid photo bleaching.

Reagents in the *therascreen* PIK3CA RGQ PCR Kit are diluted optimally and no further purification or treatment is required prior to their use.

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

Specimen Storage and Handling

Specimen handling: Tissue

The *therascreen PIK3CA RGQ PCR Kit* is for use with gDNA extracted from FFPE tumor tissue resected specimens and core needle biopsy (CNB) specimens collected from breast cancer patients. Tumors are heterogeneous in terms of both genotype and phenotype. Mutation-positive tumors can contain wild-type DNA and similarly histology can show regions of non-tumor tissue.

To prepare tissue specimens for DNA extraction:

- Using standard materials and methods, fix the tissue specimen in 10% neutral buffered formalin (NBF) and embed the tissue specimen in paraffin. Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides.
 - Use a trained individual (e.g., a pathologist) to assess a Hematoxylin & Eosin (H&E) stained section for tumor content and effective tumor area (ETA). Mark the stained slide to determine the region of interest (ROI). Use serial sections for DNA extraction.
- Note:** The stained sections must not be used for DNA extraction.
- Scrape excess paraffin away from the tissue using a fresh, sterile scalpel and discard.

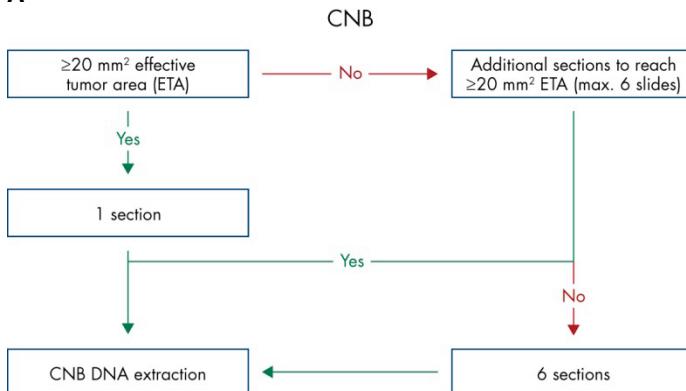
CAUTION 	Use dry scalpels. Do not perform this step in a laminar flow or fume hood.
---	--

- Scrape the tumor tissue from the slides into labeled microcentrifuge tubes using a fresh scalpel for each specimen.

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

There are two separate workflows when using FFPE tumor tissue resected specimens and FFPE CNB specimens (Figure 4).

A



B

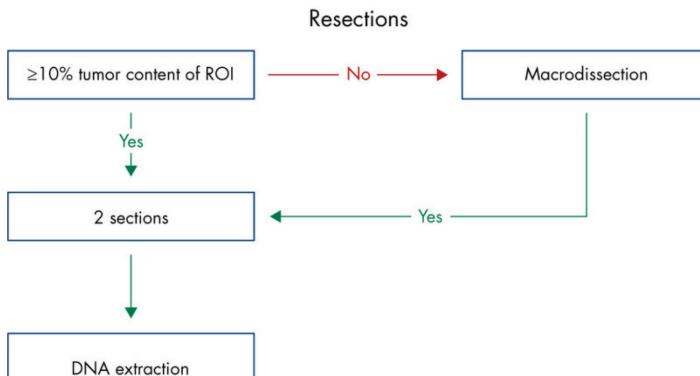


Figure 4. Clinical specimen purification workflow to be used with the *therascreen PIK3CA RGQ PCR Kit*. A: FFPE CNB. B: FFPE tumor tissue resected specimens.

Specimen handling: Plasma

The *therascreen PIK3CA RGQ PCR Kit* is for use with DNA isolated from K₂EDTA anticoagulated plasma specimens of breast cancer patients. All plasma specimens should be treated as potentially hazardous.

Whole peripheral venous blood collected in K₂EDTA blood collection tubes must be processed to obtain plasma within four hours of blood collection. Failure to do so may result in genomic DNA contamination of the sample. For further information on the isolation of plasma from whole blood, refer to Appendix A of the *QIAamp DSP Circulating Nucleic Acid Kit Handbook*.

Plasma specimens should be stored at -80°C. All frozen plasma specimens must be equilibrated to room temperature before use.

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

Specimen storage

Prior to DNA extraction FFPE blocks and slides should be stored at room temperature (15–25°C) and plasma should be stored at -80°C. DNA may be stored following extraction, prior to testing. Table 2 and Table 3 provide guidance on the maximum recommended storage times and conditions for specimens and DNA following extraction.

Table 2. Recommended storage times for gDNA extracted from FFPE tissue

Storage	Maximum recommended storage time
Freezer (-30 to -15°C)	5 weeks
Refrigerator (2–8°C)	1 week
Freezer (-80°C)	33 months

Table 3. Recommended storage conditions and times for plasma and ctDNA extracted from plasma

Specimen	Storage	Maximum recommended storage time
Plasma	Freezer (-80°C)	11 months
Extracted ctDNA	Freezer (-30 to -15°C)	4 weeks

Procedure

DNA extraction from FFPE specimens

DNA should be extracted using the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404).

Note: The *therascreen PIK3CA RGQ PCR Kit* has been developed using DNA extracted using the QIAamp DSP DNA FFPE Tissue Kit. Do not use any other DNA extraction product.

Carry out the DNA extraction according to instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook* noting the following:

- Use the number of slides and elution volumes as recommended in the sections below (“FFPE tissue resection (RES) specimens” and “FFPE CNB specimens” on page 28 of this handbook).
- If, after the first centrifugation, the tissue is not pelleted, perform an additional centrifugation.
- Make sure to use molecular biology grade ethanol* for all required steps.
- After ethanol removal, incubate the open tube at 15–40°C for 10 minutes to allow for any residual ethanol to evaporate.

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

FFPE tissue resection (RES) specimens

- If RES specimens have ≥10% tumor content in the region of interest (ROI), scrape the entire tissue area from two sections (4–5 µm) into labeled microcentrifuge tubes using a fresh scalpel for each specimen. If specimens have <10% tumor content in the ROI, perform macrodissection and scrape only the tumor ROI from two sections into labeled microcentrifuge tubes using a fresh scalpel for each specimen.
- Proteinase K digestion must be performed for 1 hour for resected tissue specimens.
- For RES specimens, purified gDNA must be eluted in 120 µl Buffer ATE (provided in the QIAamp DSP DNA FFPE Tissue Kit) after 10 minutes incubation on the column.

FFPE CNB specimens

- For CNB specimens use an adequate number of 4–5 µm sections to obtain the minimum required effective tumor area (ETA) of 20 mm² from a maximum of six sections. Use the minimum number of sections possible (1–6) to achieve 20 mm² ETA.
- For specimens where 20 mm² ETA cannot be met with the maximum of six sections, proceed with testing using six sections.
- Proteinase K digestion must be performed for 1 hour for CNB specimens.
- For CNB specimens, purified genomic DNA must be eluted in 70 µl Buffer ATE (provided in the QIAamp DSP DNA FFPE Tissue Kit), after 10 minutes incubation on the column.

DNA extraction from plasma specimens

DNA should be extracted using the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) with the stipulations described below for purifying ctDNA from plasma specimens.

Note: The *therascreen PIK3CA RGQ PCR Kit* has been developed using DNA extracted with the QIAamp DSP Circulating Nucleic Acid Kit. Do not use any other DNA extraction product.

Carry out the DNA extraction according to instructions for the "Classic Protocol" in the *QIAamp DSP Circulating Nucleic Acid Kit Handbook* noting the following:

- The starting volume of plasma is 2 ml.
- Where 2 ml is not available, adjust the volume to 2 ml using phosphate buffered saline (PBS).
- All centrifugation steps are carried out at room temperature (15–25°C).
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during the protocol steps.
- Proteinase K volume should be 250 µl.
- Purified ctDNA must be eluted in 70 µl Buffer AVE (provided in the QIAamp DSP Circulating Nucleic Acid Kit).
- The QIAamp DSP Circulating Nucleic Acid Kit must only be used manually.
- Make sure to use molecular biology grade ethanol* for all required steps
- Store purified ctDNA at –30 to –15°C.

Note: All assays in the *therascreen PIK3CA RGQ PCR Kit* generate short PCR products. However, the *therascreen PIK3CA RGQ PCR Kit* will not work with heavily fragmented DNA. The extracted DNA should be within the working Control C_T range (≥ 24.68 and ≤ 31.68) for the sample to be valid.

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

Detection of *PIK3CA* mutations

This protocol is for the detection of *PIK3CA* mutations.

Important points before starting

- Up to 24 samples can be assessed over four runs using the PIK3CA Reaction Mix available in each kit. The optimal use is four runs, with each run containing a maximum of six samples. Smaller sample batch sizes will mean that fewer samples can be tested with each *therascreen* PIK3CA RGQ PCR Kit.
- The sample must be tested using all reaction mixes provided in the *therascreen* PIK3CA RGQ PCR Kit.
- It is not possible to analyze mixed batches of samples derived from both tissue and plasma specimens in the same PCR run; PCR batches must consist of entirely tissue-derived samples or entirely plasma-derived samples.
- Do not vortex the *Taq* DNA Polymerase (Tube *Taq*) or any mix containing *Taq* DNA Polymerase, as this may inactivate the enzyme.
- Pipet *Taq* DNA Polymerase by carefully placing the pipette tip just under the liquid surface to avoid the exterior of the tip being coated in excess enzyme.

Things to do before starting

- Ensure that runs are performed using the Rotor-Gene AssayManager v2.1, Gamma Plug-in and either the “*therascreen_PIK3CA_FFPE*” Assay Profile (tissue specimens) or “*therascreen_PIK3CA_Plasma*” Assay Profile (plasma specimens). Ensure that the relevant software is installed before first use of the Rotor-Gene Q MDx 5plex HRM instrument and follow appropriate instructions on run start and data analysis (“Performing a *PIK3CA* mutation analysis run” on page 36).

- Before each use, all reagents, including *Taq* DNA Polymerase (Tube *Taq*), and DNA samples must be thawed completely for 1 hour (and up to a maximum of 4.5 hours) at room temperature (15–25°C), mixed by inverting 10 times, and centrifuged briefly to collect the contents at the bottom of the tube.
- Ensure that the PCR loading block is appropriately decontaminated (see “General precautions”, page 20) and dry.

Procedure

1. Thaw all reaction mixes, Water for No Template Control, *Taq* DNA Polymerase, PIK3CA Positive Control and DNA samples at room temperature (15–25°C) for a minimum of 1 hour and up to a maximum of 4.5 hours.
 2. After 1 hour, mix all reagents thoroughly by inverting each tube 10 times to avoid localized concentrations of salts. Centrifuge all reagents briefly to collect the contents at the bottom of the tube.
- Note:** Do not vortex the *Taq* DNA Polymerase (Tube *Taq*) or any mix containing *Taq* DNA Polymerase, as this may inactivate the enzyme.
3. Label six microcentrifuge tubes (not provided) according to Table 4. Prepare sufficient master mixes (control and mutation reaction mixes) plus *Taq* DNA Polymerase for the DNA samples, one PIK3CA Positive Control reaction and one No Template Control reaction, according to the volumes in Table 4.

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

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

Table 4. Preparation of assay master mixes

Reaction mix tube	Volume of reaction mix ($n^* + 3$)	Volume of Taq DNA polymerase ($n^* + 3$)
Tube RM 1	19.83 μ l \times ($n + 3$)	0.17 μ l \times ($n + 3$)
Tube RM 2	19.83 μ l \times ($n + 3$)	0.17 μ l \times ($n + 3$)
Tube RM 3	19.83 μ l \times ($n + 3$)	0.17 μ l \times ($n + 3$)
Tube RM 4	19.83 μ l \times ($n + 3$)	0.17 μ l \times ($n + 3$)
Tube RM 5	19.83 μ l \times ($n + 3$)	0.17 μ l \times ($n + 3$)
Tube RM 6	19.83 μ l \times ($n + 3$)	0.17 μ l \times ($n + 3$)

* n = number of DNA samples. The value n should not exceed six as six is the maximum number of samples that can fit on a run. Three extra reactions are included to ensure sufficient coverage for the PCR setup and the controls.

4. Cap the tube for the master mix and invert 10 times to thoroughly mix the master mix.
Briefly centrifuge to ensure the mix is at the bottom of the tube.
5. Immediately after the master mixes are ready, place the appropriate number of PCR 4-strip tubes (each strip has four tubes; PCR 4-strip tubes not provided) in the loading block according to the layout in Table 4. Do not cap the strip tubes. Immediately add 20 μ l of the appropriate master mix to each PCR strip tube.

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

Note: See Table 4 for the tube layout while setting up the reaction mixes.

Table 5. Run layout in the loading block for detection of PIK3CA mutations

Assay	Controls		Sample number						
	PC	NTC	1	2	3	4	5	6	7
Tube RM 1	1	9	17	25	33	41	49	57	E
Tube RM 2	2	10	18	26	34	42	50	58	E
Tube RM 3	3	11	19	27	35	43	51	59	E
Tube RM 4	4	12	20	28	36	44	52	60	E
Tube RM 5	5	13	21	29	37	45	53	61	E
Tube RM 6	6	14	22	30	38	46	54	62	E
E	E	E	E	E	E	E	E	E	E
E	E	E	E	E	E	E	E	E	E

Note: Each tube should contain a total reaction volume of 25 µl (20 µl master mix prepared according to Table 4, plus 5 µl NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position. E: Empty.

6. Immediately add 5 µl Water for No Template Control to the NTC tubes (tube positions 9–14) and cap the tubes.
7. Add 5 µl of each DNA sample to the sample tubes and cap the tubes immediately after adding each sample to avoid sample-to-sample cross-contamination.
8. Add 5 µl PIK3CA Positive Control to the PC tubes (tube positions 1–6) and cap the tubes.
9. Using a permanent marker, mark the caps of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q MDx 5plex HRM instrument.
10. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 5 and Figure 5). Take extra care to ensure that tubes will be 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 5plex HRM instrument is maintained.

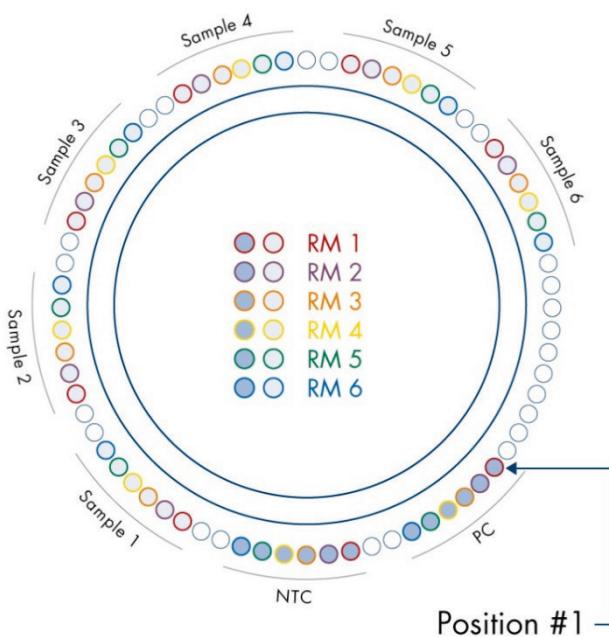
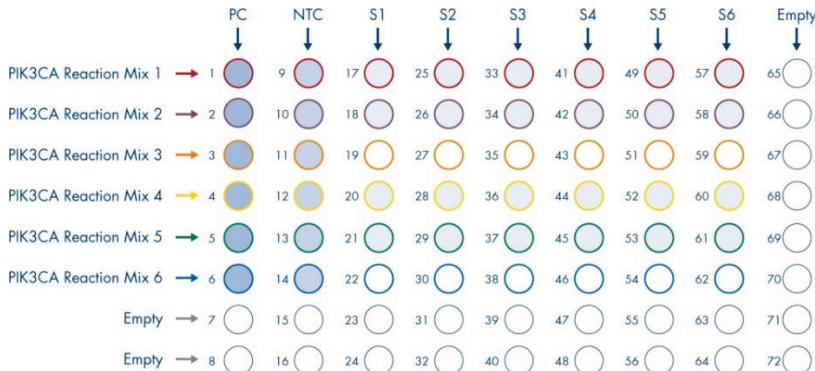


Figure 5. Plate and rotor setup for an experiment with the *therascreen* PIK3CA RGQ PCR Kit. PC: Positive Control. S: DNA sample. NTC: No-template control (water).

CAUTION

Tubes must be inserted into the rotos as indicated in Figure 5 as the automated analysis set in the Assay Profile is based on this organization. If a different layout is used, aberrant results will be obtained.

11. Immediately place the 72-well rotor into the Rotor-Gene Q MDx 5plex HRM instrument. Ensure that the locking ring (supplied with the Rotor-Gene Q MDx 5plex HRM instrument) is placed on top of the rotor to secure the tubes during the run and instrument lid is closed.
12. To start the run follow the instructions given in "Performing a PIK3CA mutation analysis run", next section.

Performing a PIK3CA mutation analysis run

13. Double-click the Rotor-Gene AssayManager v2.1 icon on the desktop of the laptop connected to the Rotor-Gene Q MDx 5plex HRM instrument.
14. The “Setup” environment appears by default. Click **New manual worklist** to create a new worklist (Figure 6).

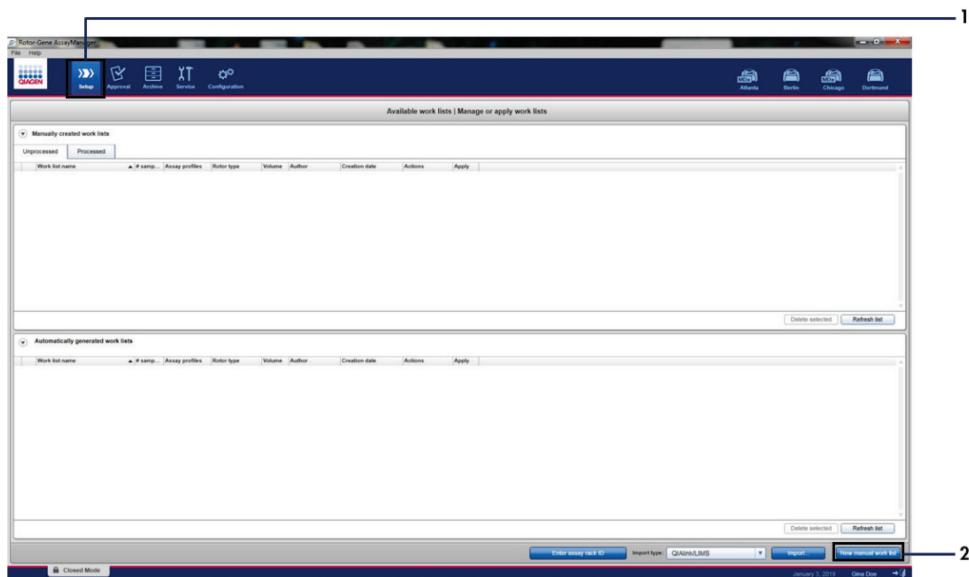


Figure 6. Setting up new manual worklist. 1 = “Setup” tab, 2 = “New manual work list”.

15. Select the "Assays" tab on the left-hand side of the main window. Depending on the sample type, click **therascreen_PIK3CA_FFPE** Assay Profile for tissue samples or **therascreen_PIK3CA_Plasma** Assay Profile for plasma samples from the list of available assay profiles and click the blue arrow to select the assay profile. If the assay profile name is truncated, point to the assay profile to see the full name (Figure 7).

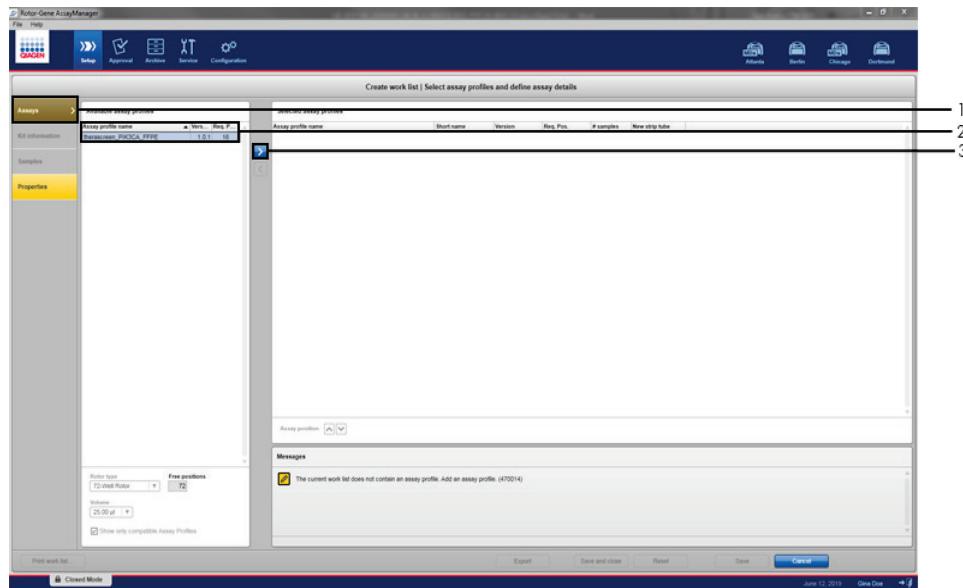
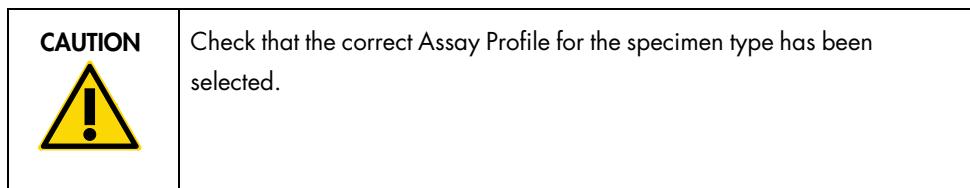


Figure 7. Setting up new manual worklist: Choosing assay profile name. 1 = "Assays" tab, 2 = Available assay profiles with "therascreen_PIK3CA_FFPE" or "therascreen_PIK3CA_Plasma" selected, 3 = Select the assay profile.

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

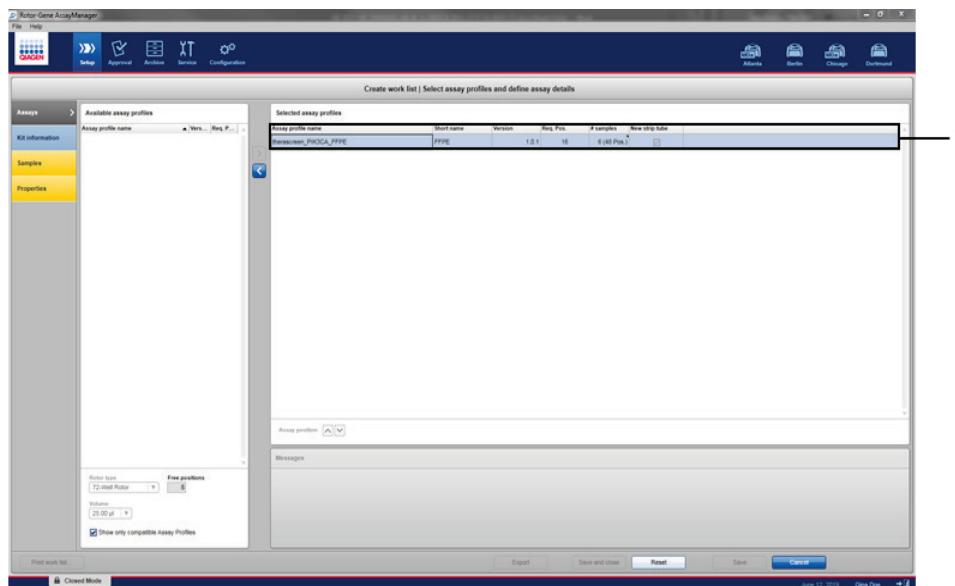


Figure 8. Create work list main window. 1 = Add the number of samples.

17. Click on the “Kit information” tab. Select **Enter kit information manually** and enter the following kit information (Figure 9):

- Kit bar code
- Material number
- Lot number
- Kit expiry date

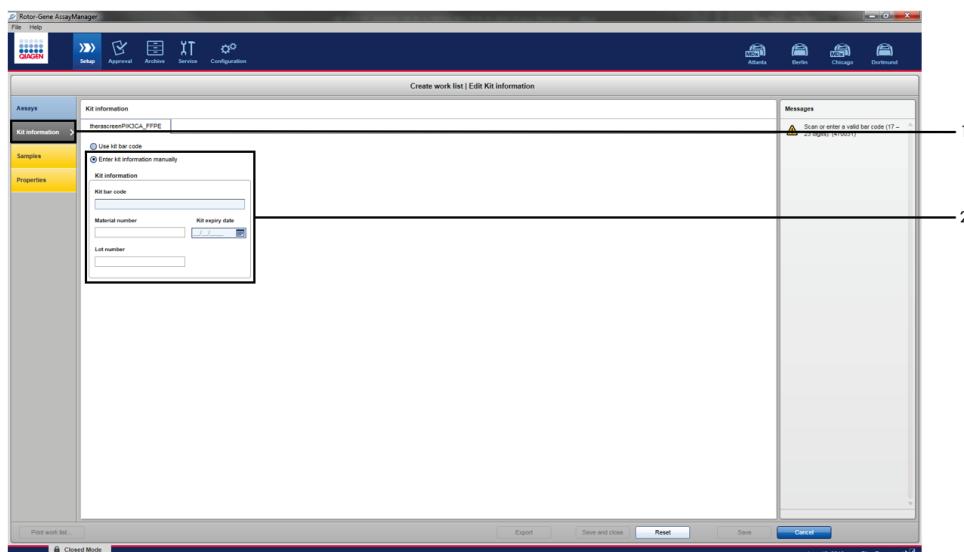


Figure 9. Create work list main window. 1 = “Kit information” tab, 2 = Enter the kit information.

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

Note: Ensure correct sample names are entered before starting the Rotor-Gene AssayManager run.

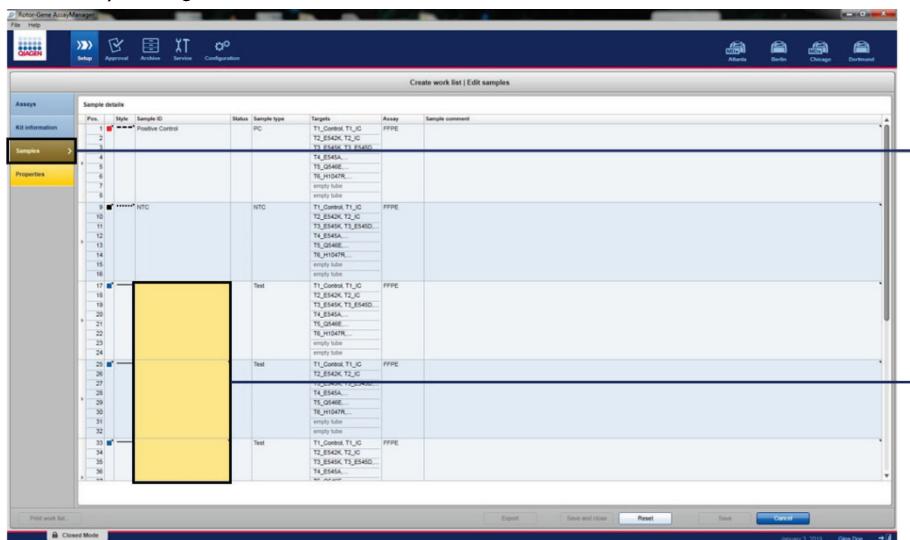


Figure 10. Create work list main window. 1= “Samples” tab, 2 = Entering sample names.

19. Click the “Properties” tab and enter the name of the worklist. After the worklist name has been entered, ensure that the **is editable** and **work list is complete** checkboxes are checked. Click **Apply** in the bottom right-hand corner to apply the worklist. A new window will appear (Figure 11).

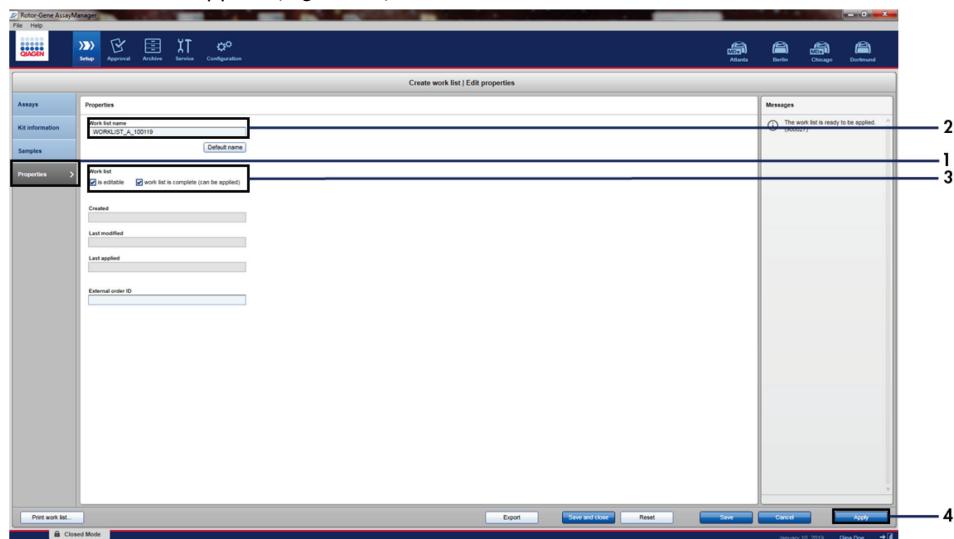


Figure 11. Create work list main window. 1 = “Properties” tab, 2 = Entering worklist name, 3 = Select “is editable” and the “work list is complete”, 4 = “Apply”.

20. Enter the experiment name into the **Experiment name** field. Select a cycler from the list of available cyclers and ensure that the **Ring attached** checkbox is checked (Figure 12). Once all steps have been performed, click **Start run**. The RGQ icon at the top left of the screen will turn green to indicate that the run has started.

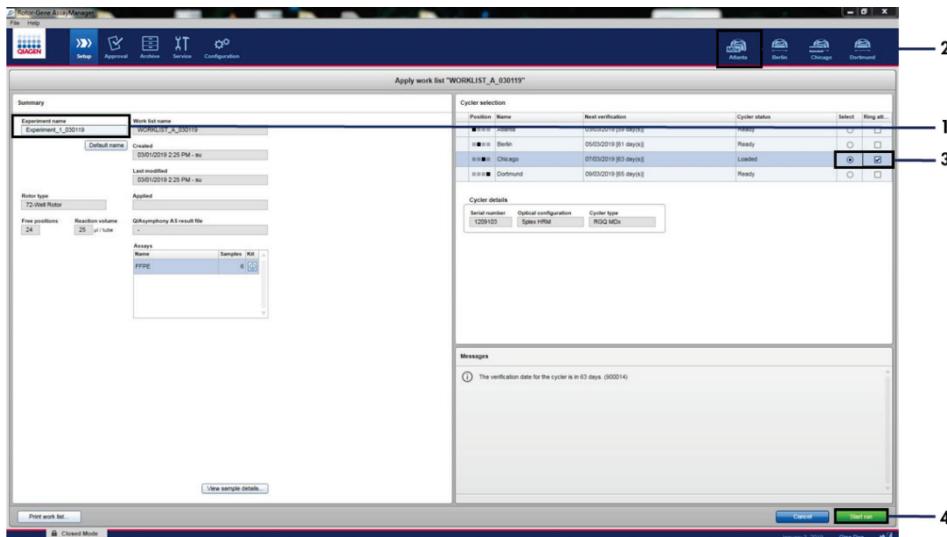


Figure 12. Applying work list and run start. 1 = Enter experiment name, 2 = Instrument selection, 3 = Ensure that "Ring attached" is selected, 4 = Start run.

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

Example cycler icons are shown in Figure 13.

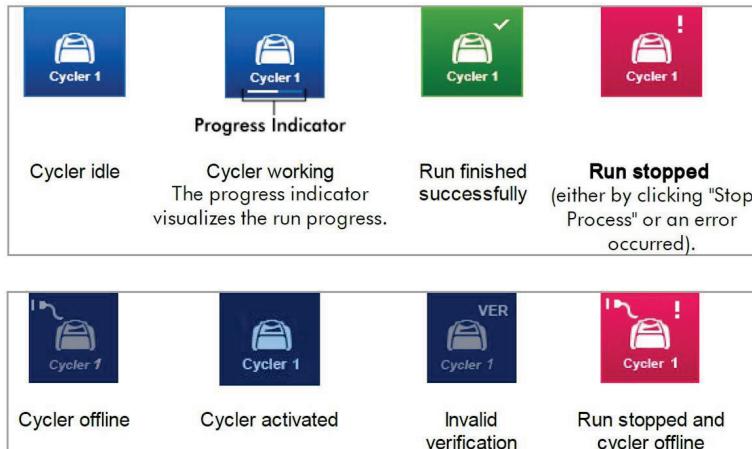


Figure 13. Cycler icons that may be displayed.

21. Once the run is complete, click **Finish run**. The “Release and go to approval” 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.

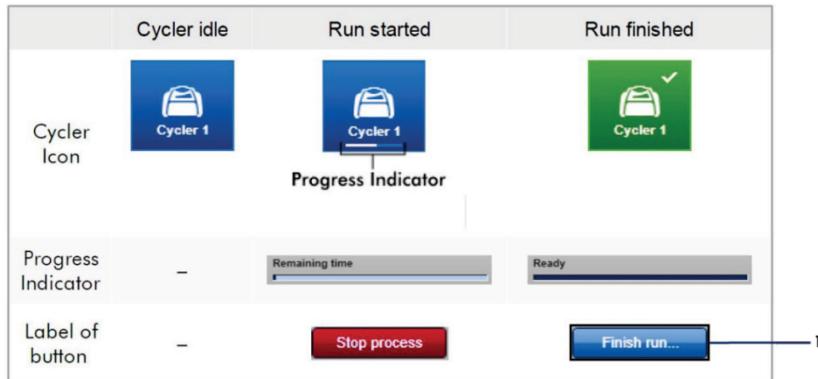


Figure 14. Finishing a run. 1 - "Finish run".

22. Click **Release and go to approval** to enter the “Approval” tab and release the Rotor-Gene Q instrument (Figure 15). The RGQ icon at the top-right of the screen will change from green to blue indicating that the instrument is ready to perform another run. Regardless of whether a run is successful or not, the run must be released and approved. For a list of potential failures and error codes presented in Rotor-Gene AssayManager see the *Rotor-Gene AssayManager v2.1 Core Application User Manual* and the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

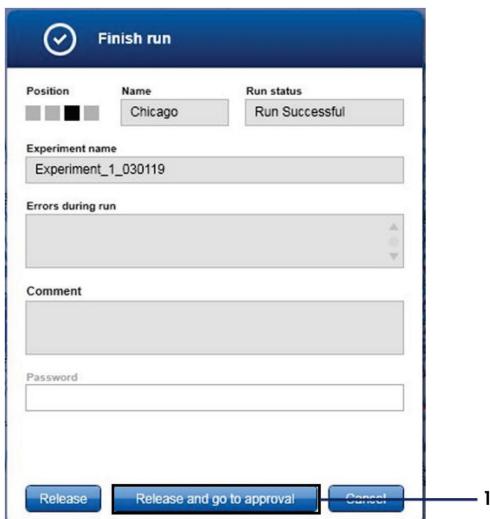


Figure 15. “Finish Run” pop up window. 1 = “Release and go to approval”.

23. Select the experiment in the “Assay selection” part of the Approval environment and click **Start approval** (Figure 16).

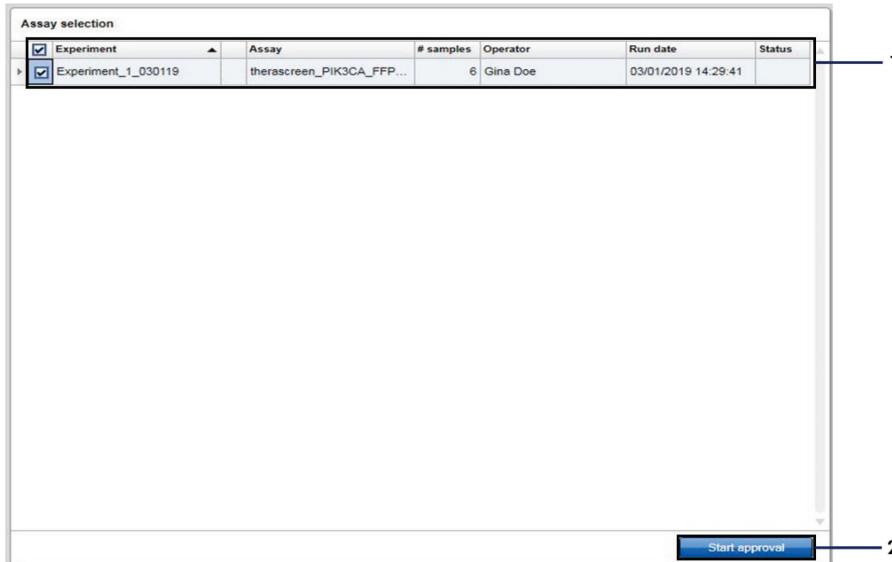


Figure 16. Starting the release process in the “Approval” environment. 1 = Assay selected to approve, 2 = “Start approval”.

“Raw data”, “Processed data”, “Experiment”, “Assay” and “Audit trail” information can be found in the “Plots and information” section (1). Assay results can be found in the “Results” section (2).

If the Positive Control and No Template Control are within an acceptable range, the “Sample Status” column will report **Valid**; otherwise an **Invalid** sample status will be reported.

If either of the run controls fails, then the run will be invalidated. All samples will be flagged as **ASSAY_INVALID**.

See “Rotor-Gene AssayManager v2.1 *therascreen PIK3CA Assay Profile flags*” (page 52) for instructions about how to proceed.

Note: The assay profile contains all of the rules for automatic assay and sample analysis and result interpretation. The software will therefore assess the validity or invalidity of samples and controls automatically.

- Click **Release/report data**. The “Release/report data” window will open (Figure 17).

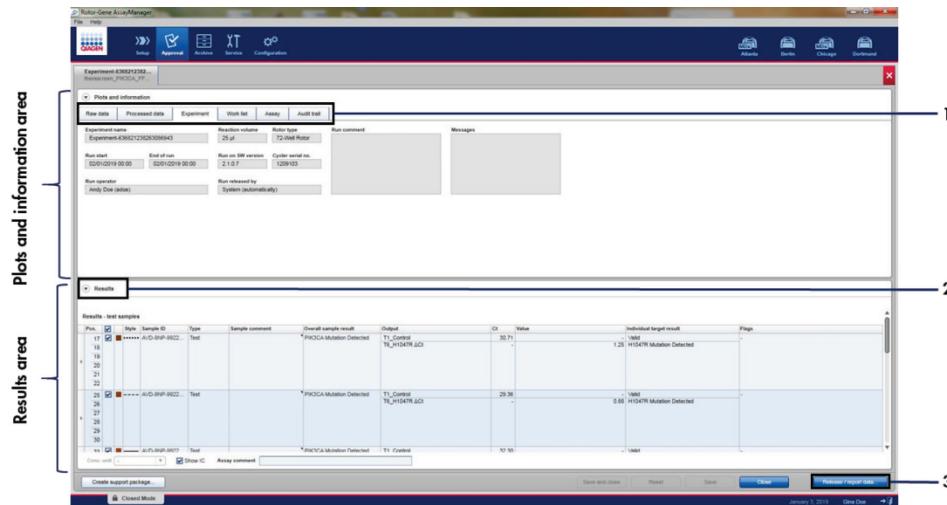


Figure 17. Example of assay results main windows. 1 = “Experiment” tab in the “Plots and information” area. 2 = Results area, 3 = “Release/report data”.

25. Click **OK** to save the experiment to the archive and create a LIMS output and run report (Figure 18). 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” tab.

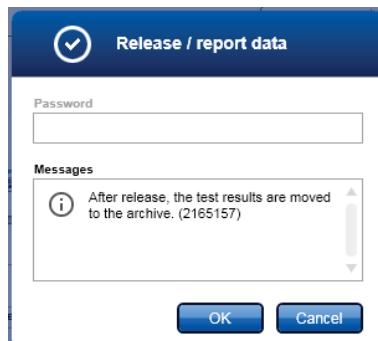


Figure 18. Example of “Release/report data” window.

26. To view an experiment stored in the experiment archive, click **Archive** 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 19).

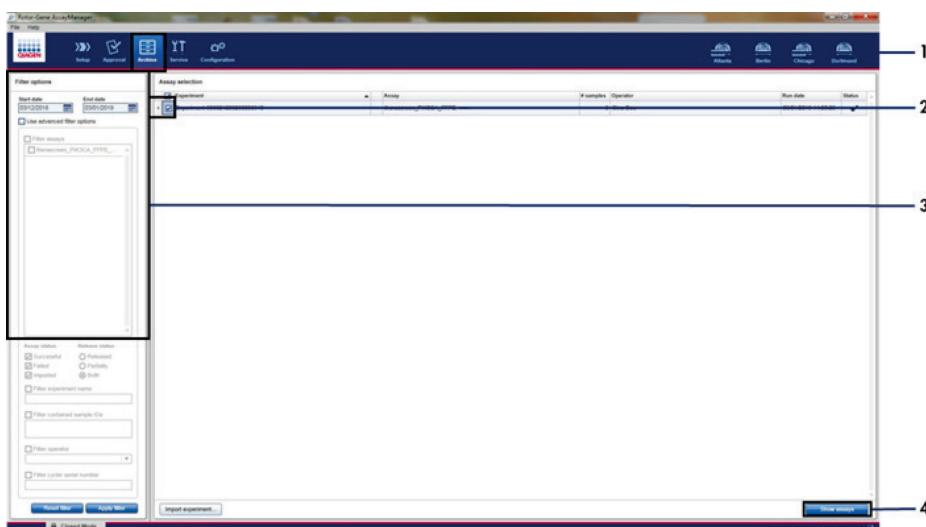


Figure 19. Example of “Experiment Archive” main window. 1 = “Archive” tab, 2 = Search options, 3 = Selecting experiment name, 4 = “Show assays” tab.

Results

Analysis and mutation calls are performed automatically by the *therascreen PIK3CA Assay Profile* when a run is completed. The following information explains how the *therascreen PIK3CA Assay Profile* makes the analysis and mutation calls.

Analysis

The PCR cycle at which the fluorescence from a particular reaction crosses the predefined threshold value given by the *therascreen PIK3CA Assay Profile* is defined as the C_T value. C_T values indicate the quantity of specific input DNA. Low C_T values indicate higher input DNA levels and high C_T values indicate lower input DNA levels. Reactions in which fluorescence crosses the threshold value on or before this C_T value are classed as positive.

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

Assessing the samples using the different mutation reaction mixes to determine their respective C_T values allows the *therascreen PIK3CA Assay Profile* to perform a calculation to determine the ΔC_T value of the sample using the equation:

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

Based on predetermined analytical C_T and ΔC_T values, the *therascreen PIK3CA Assay Profile* qualitatively determines the mutation status of the DNA samples and reports if a sample contains a mutation(s).

The run controls (PC, NTC, and the IC) are assessed to ensure that acceptable C_T values are met and the reactions have been performed successfully.

If the sample Control C_T is below the acceptable range this means that DNA input is too high and the sample needs to be diluted as described in “Rotor-Gene AssayManager v2.1 *therascreen PIK3CA Assay Profile flags*”, page 52.

All of these assessments are performed automatically and require no manual interpretation. The system automatically checks run validity and sample validity criteria and will not report mutation status in the event of an invalid sample or invalid run.

The Rotor-Gene AssayManager v2.1 software determines the result for each biomarker target by combining all relevant analysis results according to core analysis algorithms such as normalization, sample and assay rules defined in the corresponding assay profile.

The following results may be assigned to an individual sample:

- PIK3CA Mutation Detected
- No Mutation Detected
- INVALID: If one or more flags are assigned to the sample during analysis by the Rotor-Gene AssayManager v2.1 software that are defined to set the target result to “INVALID”.

Note: If an error has occurred during the run, the samples in the Rotor-Gene Q MDx 5plex HRM must be disposed of and not be retested.

Rotor-Gene AssayManager v2.1 *therascreen PIK3CA* Assay Profile flags

All possible flags corresponding to the Rotor-Gene AssayManager v2.1 Gamma Plug-in are listed in the *Rotor-Gene AssayManager v2.1 Gamma Plug-in User Manual*.

Table 6 lists the possible flags that may be generated by the *therascreen PIK3CA* Assay Profiles, their meaning, and actions to be taken.

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

For example:

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

Table 6. Software flags used by the PIK3CA Assay Profiles

Flag	Meaning	Action
IC_ABOVE_ACCEPTED_RANGE	Invalid run. IC value above the specification range in PC or NTC tubes.	Repeat run.
(PC)_ABOVE_ACCEPTED_RANGE	Invalid sample. IC in sample above the specification range.	Retest the sample once; after retest if sample IC C_T is still above acceptable range, re-extract the sample. If the sample IC is still above the acceptable range after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.
(PC)_BELOW_ACCEPTED_RANGE	Invalid run. PC below the specification range.	Repeat run.
IC_BELOW_ACCEPTED_RANGE	Invalid run. IC below the specification range in PC or NTC tubes.	Repeat run.
	Invalid sample. IC in sample below the specification range	Retest the sample once; after retest if sample IC C_T is still below acceptable range, re-extract the sample. If the sample IC is still below the acceptable range after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.
UNEXPECTED_CT_VALUE	Invalid run. C_T value has been detected in NTC.	Repeat run.
NO_CT_VALUE	Invalid PC or IC. No C_T value for PC in PC tubes or for IC in PC and NTC tubes.	Repeat run.
	Invalid sample. No C_T value in sample.	Retest the sample once; after retest if there is still no sample IC C_T , re-extract the sample. If there is still no sample IC after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.

Table continued on next page

Table 6. Software flags used by the PIK3CA Assay Profiles, continued

Flag	Meaning	Action
DNA_INPUT_TOO_HIGH	Invalid sample. Sample Control C _T value below the Control working range.	Sample is too concentrated and must be diluted. Follow instructions in "Control C _T value", page 54.
ABOVE_ACCEPTED_RANGE	Invalid sample. Sample Control C _T value above the Control working range.	Retest the sample once; after retest if the Control C _T value is still above the Control working range, re-extract the sample. If the Control C _T value is still above the Control working range after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.
T1_CONTROL_NO_CT_VALUE	Invalid sample. No C _T value for sample in sample control tubes.	Retest the sample once; after retest if sample has no C _T , re-extract the sample. If the sample still has no C _T after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.

Note: If a retested sample is invalid for a different reason upon repeat, this is still classed as a second repeat and a re-extraction of the sample should be performed.

Control C_T value

There are two possible flags for invalid sample due to Control C_T value:

- **DNA_INPUT_TOO_HIGH:** Sample is too concentrated and will overload the mutation assays. In order to obtain a valid sample result, the sample must be diluted. Samples should be diluted on the basis that diluting by half will increase the C_T by 1. Samples should be diluted using the water provided in the kit (Water for Dilution [Dil.]).

To calculate the required Control C_T shift (X_R) and estimate the dilution factor required (Table 7):

$$X_R = 25 - X \text{ (FFPE specimens)}$$

$$X_R = 27 - X \text{ (plasma specimens)}$$

where 25 (for FFPE specimens) or 27 (for plasma specimens) is given the target Control C_T for the diluted sample and X is an actual Control C_T of sample to be diluted.

If X is not a whole number then round up to the next whole number, e.g., 2.1 is rounded up to 3.0. This value is X_R. Obtain the dilution factor required from Table 7.

Table 7. Calculation of dilution factor

X _R	Dilution factor	Sample ratio	Dil. ratio
1	2-fold	1	1
2	4-fold	1	3
3	8-fold	1	7
4	16-fold	1	15
5	32-fold	1	31
6	64-fold	1	63
7*	128-fold	1	127
8*	256-fold	1	255

* For plasma only.

- **ABOVE_ACCEPTED_RANGE and T1_CONTROL_NO_CT_VALUE:** The quantity of DNA is insufficient for mutation analysis. Retest the sample where sufficient DNA eluate is available (>30 µl). If the quantity of DNA is still insufficient upon retest, re-extract from fresh FFPE sections or a fresh plasma specimen. If not possible, the sample should be reported as indeterminate.

Performance Characteristics: Tissue Specimens

Analytical performance: Tissue specimens

The specific performance characteristics of the *therascreen* PIK3CA RGQ PCR Kit were determined in studies using FFPE tissue specimens collected from breast cancer patients and 12 FFPE human cell-line specimens (FFPE cell-line specimens) that harbor known *PIK3CA* mutations detected by the assay, plus one *PIK3CA* wild-type specimen (i.e., no mutations claimed to be detected by the *therascreen* PIK3CA RGQ PCR Kit in exons 7, 9, and 20).

Limit of blank (LoB): Tissue specimens

The LoB is defined in CLSI guideline EP17-A2 as “the highest measurement result that is likely to be observed (with a stated probability) for a blank sample”. For the *therascreen* PIK3CA RGQ PCR Kit this is the data point that corresponds to the upper 95% percentile in the mutation-negative samples. The LoB was determined by the analysis of 56 individual clinical wild-type FFPE specimens (30 RES specimens and 26 CNB specimens) tested in duplicate per sample for each of three *therascreen* PIK3CA RGQ PCR Kit lots generating 336 data points total. The LoB values for each of the mutation assays (in terms of ΔC_t) detected by the *therascreen* PIK3CA RGQ PCR Kit were verified to be above the ΔC_t cutoff values determined for each of the assays and are summarized below along with the false-positive call rates obtained.

Table 8. Summary of LoB results

Exon	Mutation	Base change	LoB (ΔC_t value)	False positive call rate (%)
7	C420R	1258T>C	7.57	0.94
9	E542K	1624G>A	5.09	1.88
	E545A	1634A>C	13.03	0.00
	E545D	1635G>T	9.19	0.31
	E545G	1634A>G	13.03	0.00
	E545K	1633G>A	6.74	1.57
	Q546E	1636C>G	13.03	0.00
	Q546R	1637A>G	8.72	0.00
20	H1047L	3140A>T	12.63	0.94
	H1047R	3140A>G	9.80	1.25
	H1047Y	3139C>T	7.61	0.63

Limit of detection (LoD): Tissue specimens

A study was conducted to determine the LoD of each of the 11 *PIK3CA* mutations. LoD was defined as the lowest amount of mutant DNA in a background of wild-type DNA at which a mutant sample will provide mutation positive results in 95% of the test results (C_{95}). The LoDs for the 11 *PIK3CA* mutation assays of the *therascreen PIK3CA RGQ PCR Kit* are reported as MAF. To determine the LoD for each mutation, breast cancer FFPE clinical specimens or FFPE cell-line DNA with different percentages of mutation were prepared at low DNA input by serially diluting in an FFPE clinical wild-type background. For each *PIK3CA* mutation, the percentage of correct calls was assessed across dilution levels using three different *therascreen PIK3CA RGQ PCR Kit* lots with 24 replicates tested per kit lot per five to six MAF levels. The LoD for each assay was calculated using a 'probit' method (Table 9). The final LoD value for each mutation was determined as the highest value (in terms of MAF) across all *therascreen PIK3CA RGQ PCR Kit* lots. To verify the LoD, mutation samples at the determined LoD were tested and the positive test rate verified in the repeatability and reproducibility study.

Table 9. LoD for tissue specimens established using low DNA input samples derived from FFPE clinical specimens and FFPE cell-line specimens

Exon	Mutation	COSMIC* ID	Base change	LoD (% MAF)
7	C420R	757	1258T>C	2.41 [†]
9	E542K	760	1624G>A	5.47 [‡]
	E545A	12458	1634A>C	3.54 [†]
	E545D	765	1635G>T	2.69 [‡]
	E545G	764	1634A>G	4.98 [‡]
	E545K	763	1633G>A	4.13 [‡]
	Q546E	6147	1636C>G	4.50 [†]
	Q546R	12459	1637A>G	6.08 [‡]
20	H1047L	776	3140A>T	2.56 [‡]
	H1047R	775	3140A>G	3.13 [‡]
	H1047Y	774	3139C>T	14.04 [†]

MAF: Mutant allele frequency.

* COSMIC: Catalogue of somatic mutations in cancer: <https://cancer.sanger.ac.uk/cosmic>.

† LoD values were established using DNA from cell-line specimens.

‡ LoD values were established using DNA from clinical specimens.

Genomic DNA input range: Tissue specimens

The *therascreen* PIK3CA RGQ PCR Kit does not use a specific concentration of DNA as determined by spectrophotometry. DNA input is based on the Control Reaction C_T result, which is used to indicate that there is sufficient amplifiable DNA present in the sample. The Control C_T working range was determined using a total of 20 wild-type FFPE clinical specimens generating 107 data points. The Control C_T working range was set using calculated tolerance intervals. The control reaction C_T range was established as 23.23 to 33.38 C_T.

ΔC_T cutoff values: Tissue specimens

The assay cutoff value is a specific ΔC_T value used to determine whether a sample is classed as positive or negative for a PIK3CA mutation. Samples that generate ΔC_T values at or below the cutoff are classified as PIK3CA mutation positive (i.e., PIK3CA Mutation Detected) and ΔC_T values generated above the cutoff are classified as PIK3CA mutation negative (i.e., No Mutation Detected). A mixture of cell-line, clinical specimens, and pre-extracted cell-line DNA were used to establish the cutoff values of each mutation. The cutoffs were chosen with respect to the following parameters: false-positive fraction, false-negative fraction and assay sensitivity.

The cutoff for each assay within the *therascreen* PIK3CA RGQ PCR Kit is shown in Table 10.

Table 10. Cutoff values for each mutation assay when testing DNA from tissue specimens

Assay	Cutoff value (ΔC_T)
C420R	≤ 6.0
E542K	≤ 4.8
E545A	≤ 10.0
E545D	≤ 7.5
E545G	≤ 9.5
E545K	≤ 6.5
Q546E	≤ 10.0
Q546R	≤ 7.0
H1047L	≤ 10.0
H1047R	≤ 7.0
H1047Y	≤ 6.2

Effect of DNA input on ΔC_T values (linearity): Tissue specimens

The DNA input level is defined as the total quantity of amplifiable DNA in a sample as determined by the C_T values from the PIK3CA control reaction. To demonstrate that the performance of the *therascreen* PIK3CA RGQ PCR Kit is consistent across the control reaction C_T range [23.23 to 33.38], a 9-level serial dilution with varying DNA input levels with the upper and lower levels being outside of the Control Reaction C_T working range [23.23–33.38 C_T], were evaluated with mutation-positive samples. Three different specimen types were used in this study: clinical FFPE resection specimens, cell-line FFPE specimens and gDNA pre-extracted from cell lines. The MAFs were held constant while DNA input was varied. The target C_T values for dilutions levels 1 and 9, for each mutation, were approximately 23.00 and 33.50, respectively. Both values were targeted to be outside of the control reaction C_T range.

The evaluation was performed using one *therascreen* PIK3CA RGQ PCR Kit lot with three replicates tested per DNA level. The data was analyzed using regression analysis to determine the linear range. For the assay to be determined as linear across the DNA input range, there should be no change across the range in ΔC_T , i.e., there is no statistically significant linear, quadratic or cubic effect. Overall, the ΔC_T values measured at different total DNA input levels were consistent across the working range of the *therascreen* PIK3CA RGQ PCR Kit for mutations E542K, E545D, E545G, E545A, H1047Y, Q546E, C420R, and H1047R, i.e., these assays did not show a statistically significant p-value ($p>0.05$) for the linear, quadratic and cubic effects fitted for all models tested. The E545K, Q546R, and H1047L assays are not linear for ΔC_T across the tested DNA input range. A linear range for the E545K assay was observed between C_T 24.08 and 31.02. A linear range for the Q546R assay was observed between C_T 24.28 and 32.69. A linear range for the H1047L assay was observed between C_T 25.74 and 31.61. An investigation determined that the non-linear effects had no effect on the performance of the E545K and H1047L assays. However, an effect on the Q546R assay performance was determined; samples at LoD may be called false negative when the DNA input is high (approximately Control C_T 23); however, the probability of this occurring is extremely low, approximately 0.0052%.

Assay specificity (cross-reactivity/specificity): Tissue specimens

The *therascreen PIK3CA RGQ PCR Kit* is comprised of six separate reaction mixes: a single Control Reaction that detects a region in exon 15 of the *PIK3CA* gene and 11 mutation assays that detect *PIK3CA* mutations. There is no reaction that specifically measures the wild-type *PIK3CA* sequence at exons 7, 9, or 20. The *therascreen PIK3CA RGQ PCR Kit* "No Mutation Detected" result is inferred by the absence of any positive mutation results.

To assess whether cross-reactivity between mutations detected by the assay has been correctly accounted for in the setting of the analytical cutoff values, mutant-positive clinical specimens and cell-line specimens, were tested in duplicate using three lots of the *therascreen PIK3CA RGQ PCR Kit* at low DNA input and low MAF% and high DNA input and high MAF% (generating 240 data points total). Within this study, there was one instance of cross-reactivity between E545D and H1047R, and one instance between C420R and H1047R. There were also four instances of mutant nonspecific amplification between the high MAF sample E545A and H1047L. Overall, 6/240 data points showed mutant nonspecific amplification. The six data points showing mutant nonspecific amplification were sporadic and inconsistent with other replicates from the same sample. These results were therefore not considered to be a result of cross-reactivity. However, PCR cross-reactivity was observed between H1047L and H1047R. This cross-reactivity is unidirectional i.e., if a double H1047R and H1047L sample is seen, this will only be reported as "H1047R Mutation Detected". This rule is incorporated in to the automated "*therascreen_PIK3CA_FFPE*" Assay Profile algorithm.

Interference: Tissue specimens

Effects of necrotic tissue

To evaluate the potential interference of necrotic tissue content in breast cancer FFPE specimens on the performance of the *therascreen PIK3CA RGQ PCR Kit*, FFPE clinical specimens from SOLAR-1 with both *therascreen PIK3CA RGQ PCR Kit* and next generation sequencing (NGS) results were analyzed. A total of 180 *PIK3CA* mutant-negative specimens by NGS and 199 *PIK3CA* mutant-positive by NGS were evaluated, which included CNB and RES specimens. Percent necrosis, as identified by a pathologist, varied from 0 to 10% for mutant-negative and 0 to 20% for mutant-positive samples.

For both mutant-positive and mutant-negative FFPE specimens, 20 samples had *therascreen PIK3CA RGQ PCR Kit* results that were discordant with the expected NGS results. These results were from 17 mutant-negative and two mutant-positive samples with less than 5% necrotic content, and one mutant-negative sample with less than 10% necrotic content; thus, it is unlikely that necrosis was the reason for the discordant results. The results support the use of the *therascreen PIK3CA RGQ PCR Kit* with breast cancer FFPE specimens with necrotic tissue content up to 20%.

Effects of hemoglobin and exogenous substances

The effect of potential interfering substances introduced from the FFPE extraction kit (an exogenous substance) or from the sample itself (hemoglobin) on assay performance were measured by comparison of ΔC_t between interferent spiked and control spiked extracts of each mutant and comparison of the correct calls for wild-type DNA samples.

The exogenous substances present in the DNA extraction process tested were:

- Paraffin wax
- Xylene
- Ethanol

- Buffer ATL
- Proteinase K
- Buffer AL
- Buffer AW1
- Buffer AW2

Samples to be spiked with exogenous interferents were first normalized to C_T 30.00 and then diluted with wild-type (also normalized to C_T 30.00) to give the ΔC_T expected at a MAF representing 3x LoD. Samples spiked with hemoglobin (endogenous interferent) during the extraction process were not normalized to C_T 30.00 or diluted to 3x LoD prior to mutation assessment, but used immediately following extraction. This was to avoid removing any variability that may have been introduced by the interferent.

The study required the preparation of a test sample set and a blank sample set (Buffer ATE for exogenous substances and water for hemoglobin). The test sample set included all mutant and wild-type samples spiked with an interferent. The blank sample set included mutant and wild-type samples spiked with an appropriate control substance. Samples tested with hemoglobin were spiked during the extraction process to reflect what would be introduced via the FFPE sample. The test concentration of hemoglobin and the estimated tissue volume used in the extraction process were based on CLSI guidelines (CLSI EP7-A2, Appendix D, 2005, Interference Testing in Clinical Chemistry; Approved Guideline). The recommended testing concentration of hemoglobin given in EP07-A, Appendix D, 2005 is 2 mg/ml. Samples tested with potential exogenous interferents were spiked following normalization to C_T 30.00 and dilution to 3x LoD at a concentration representing the highest (worst-case) feasible level of the interfering substance carryover into a sample (10x concentration). In total, six replicates of each sample/interferent combination were tested with one *therascreen* PIK3CA RGQ PCR Kit lot. All mutation calls in both mutant and wild-type samples were as expected. Where a significant difference was observed between the spiked and control samples, this was within acceptable intermediate precision of the assay and was, therefore within the inherent

variability of the assay. The results demonstrated that these substances did not interfere with the call results of the *therascreen PIK3CA RGQ PCR Kit*.

Lot interchangeability: Tissue specimens

The *therascreen PIK3CA RGQ PCR System* utilizes the QIAamp DSP DNA FFPE Tissue Kit, for isolation of DNA and the *therascreen PIK3CA RGQ PCR Kit* for the amplification of DNA and detection of *PIK3CA* mutation status. Lot-to-lot reproducibility was demonstrated using three lots of the QIAamp DSP DNA FFPE Tissue Kit and three lots of the *therascreen PIK3CA RGQ PCR Kit*. The overall percentage of correct calls across lots for all mutation-positive and wild-type samples was 96.8% (363/375).

Specimen handling: Tissue specimens

The reproducibility of the QIAamp DSP DNA FFPE Tissue Kit was examined using sections taken from 11 FFPE specimen blocks; four *PIK3CA* mutant clinical breast cancer specimens, six *PIK3CA* mutant cell-line specimens and one wild-type clinical breast cancer specimen. For each specimen, extractions were carried out in triplicate by two operators, at three sites, yielding a total of 18 data points per specimen. At each site the testing was conducted using one lot of the QIAamp DSP DNA FFPE Tissue Kit and one lot of the *therascreen PIK3CA RGQ PCR Kit* reagents. All valid mutant and wild-type specimen results yielded the expected overall mutation status result (correct call = 100%, 18/18 for each specimen). Across specific *PIK3CA* mutation calls, the proportion of correct calls was 97.92% supporting the reproducibility and repeatability for the *therascreen PIK3CA RGQ PCR Kit* at the pre-analytical step of DNA isolation.

Repeatability and reproducibility: Tissue specimens

The precision and reproducibility of the *therascreen PIK3CA RGQ PCR Kit* was investigated by testing DNA extracted from FFPE clinical breast cancer specimens for *PIK3CA* mutations E542K, E545G, E545K, H1047L, H1047R, and Q546R, and cell-line FFPE samples for *PIK3CA* mutations C420R, E545A, E545D, H1047Y, Q546E, and Q546R. Wild-type FFPE clinical breast specimens were also included in the study (Table 11).

To demonstrate repeatability, samples at two mutation levels (LoD and 3x LoD) were tested in duplicate with two runs per day, by three operators across 20 non-consecutive days resulting in 120 data points at one site (located in the United Kingdom) except for samples at LoD with E545A and Q546R *PIK3CA* mutations. Samples with E545A and Q546R mutations at LoD were evaluated for six days at one site by three operators, with two runs and four replicates for a total of 144 measurements to demonstrate repeatability. For reproducibility two runs per day were performed per operator (three operators per site) by two additional sites (both located in the USA) over 10 days to give an additional 60 data points for each additional site, except for samples at LoD with E545A and Q546R *PIK3CA* mutations. Samples at LoD with E545A and Q546R *PIK3CA* mutations were evaluated for six days for two more sites, by three operators, with two runs and four replicates for a total of 144 measurements per site, 432 in total across three sites. At each site, samples were tested using two *therascreen PIK3CA RGQ PCR Kit* lots (three lots across three sites). One to two lots of QIAamp DSP DNA FFPE Tissue Kit were used to extract DNA from FFPE specimens. Samples were prepared at low DNA input levels where a control C_t value of approximately 30 was targeted.

Mutation-positive samples were only run with the control reaction mix and the relevant reaction mix of the mutation of interest. Wild-type samples were run with all reaction mixes.

For each sample, the proportion of correct calls is shown in Table 11 for repeatability.

Table 11. Assay repeatability – proportion of correct calls for PIK3CA mutations tested in DNA samples obtained from FFPE tissue specimens

Exon	Mutation	Mutation level	Fractional proportion of valid results	Correct calls, %	Lower two-sided 95% CI
NA	Wild type	NA	108/120	90.00	83.18
7	C420R	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
9	E542K	LoD	119/119	100.00	96.95
		3x LoD	120/120	100.00	96.97
	E545A	LoD*	144/144	100.00	97.47
		3x LoD	120/120	100.00	96.97
	E545D	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
	E545G	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
20	E545K	LoD	118/120	98.33	94.11
		3x LoD	120/120	100.00	96.97
	Q546E	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
	Q546R	LoD*	139/140	99.29	96.08
		3x LoD	119/119	100.00	96.95
	H1047L	LoD	117/120	97.50	92.87
		3x LoD	120/120	100.00	96.97
	H1047R	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
	H1047Y	LoD	117/120	97.50	92.87
		3x LoD	120/120	100.00	96.97

NA: Not applicable.

* Samples at LoD with E545A and Q546R PIK3CA mutations were evaluated for six days at one site by three operators, with two runs and four replicates for a total of 144 measurements.

Table 12. Assay reproducibility – proportion of correct calls for PIK3CA mutations tested in DNA samples obtained from FFPE tissue specimens

Exon	Mutation	Mutation level	Fractional proportion of valid results	Correct calls, %	Lower two-sided 95% CI
NA	Wild type	NA	222/240	92.50	88.41
7	C420R	LoD	240/240	100.00	98.47
		3x LoD	240/240	100.00	98.47
9	E542K	LoD	237/239	99.16	97.01
		3x LoD	240/240	100.00	98.47
	E545A	LoD*	431/432	99.77	98.73
		3x LoD	240/240	100.00	98.47
	E545D	LoD	238/240	99.17	97.02
		3x LoD	240/240	100.00	98.47
	E545G	LoD	240/240	100.00	98.47
		3x LoD	240/240	100.00	98.47
	E545K	LoD	238/240	99.17	97.02
		3x LoD	240/240	100.00	98.47
	Q546E	LoD	240/240	100.00	98.47
		3x LoD	240/240	100.00	98.47
	Q546R	LoD*	421/424	99.29	97.95
		3x LoD	239/239	100.00	98.47
20	H1047L	LoD	230/240	95.83	92.47
		3x LoD	240/240	100.00	98.47
	H1047R	LoD	240/240	100.00	98.47
		3x LoD	240/240	100.00	98.47
	H1047Y	LoD	234/240	97.50	94.64
		3x LoD	240/240	100.00	98.47

NA: Not applicable.

* Samples at LoD with E545A and Q546R PIK3CA mutations were evaluated for six days across three sites, by three operators, with two runs and four replicates for a total of 144 measurements per site, 432 total.

A variance component analysis was used to estimate the standard deviation for between-kit, between-run, between-operator, between-instrument, between-day and within-run variability for repeatability and reproducibility. Across all variance components, the total standard

deviation (SD) was $\leq 1.32 \Delta C_T$ for LoD and $\leq 0.63 \Delta C_T$ for 3x LoD for all *PIK3CA* mutations tested in the reproducibility testing. Across all mutant panel members, the SD was $\leq 0.17 \Delta C_T$ for LoD and $\leq 0.16 \Delta C_T$ for 3x LoD for between-lots (lot interchangeability). The SD for within-run variability (repeatability) was $\leq 1.24 \Delta C_T$ for LoD and $\leq 0.53 \Delta C_T$ for 3x LoD.

Cross-contamination/analytical carryover: Tissue specimens

The purpose of this study was to evaluate the *therascreen PIK3CA RGQ PCR Kit* when high *PIK3CA* mutation-positive samples were tested adjacent to *PIK3CA* mutation-negative samples. This study investigated the probability of cross-contamination during the whole testing procedure (DNA extraction and subsequent testing with the *therascreen PIK3CA RGQ PCR Kit*).

This study was performed with H1047R (the mutation with highest prevalence) and wild-type FFPE cell-line specimens. Two independent sets of samples referred to as "Set A" and "Set B" were extracted following a predefined extraction matrix designed to introduce risk of sample cross-contamination. Two operators performed the extractions. A total of 18 extractions (nine per set) were carried out for the mutation positive (H1047R) samples. A total of 42 extractions (21 per set) were carried out for the wild-type samples. The extracts were assessed for mutation across ten PCR runs; five per sample set were set up consecutively by the same operator using the same equipment and Rotor-Gene Q instrument, with no other runs set up using this instrument between these runs. Extracts were tested with the control assay reaction mix (*therascreen PIK3CA RGQ PCR Kit* Tube 1) and mutation of interest (*therascreen PIK3CA RGQ PCR Kit* Tube 6).

The observed percentage of correct mutation calls for valid wild-type samples was 100%, demonstrating no cross-contamination of the wild-type samples by mutant samples sharing the same DNA extraction and run set up procedure.

Accuracy: Comparison to the analytical reference method (tissue specimens)

To demonstrate the accuracy of the *therascreen PIK3CA RGQ PCR Kit* relative to a validated NGS assay, an accuracy study was conducted using FFPE clinical specimens from breast cancer patients randomized in the SOLAR-1 trial and for which there was sufficient quantity of specimen available for testing with the NGS comparator assay. Of these 453 clinical specimens, 385 met the NGS comparator specimen requirements for tissue volume and tumor content and 379 yielded a valid result for NGS.

Samples with valid results for both NGS and the *therascreen PIK3CA RGQ PCR Kit* were analyzed with NGS as the reference to assess the positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA). These percentages, together with the corresponding two-sided 95% confidence intervals (CI), calculated using the Clopper-Pearson Exact method, are summarized in Table 13.

Table 13. Analysis of agreement for FFPE tissue specimens

Measure	Percent agreement (N)	Two-sided 95% CI
Positive percent agreement	99.0 (197/199)	96.4, 99.9
Negative percent agreement	90.0 (162/180)	84.7, 94.0
Overall percent agreement	94.7 (359/379)	92.0, 96.7

For the 20 overall mutation status discordant results, two samples with *therascreen PIK3CA RGQ PCR Kit* negative results had NGS-positive results, while 18 samples with *therascreen PIK3CA RGQ PCR Kit*-positive results gave NGS-negative results. Of the two samples with *therascreen PIK3CA RGQ PCR Kit* negative results that had NGS-positive results, both were detected by the NGS at MAF levels below the *therascreen PIK3CA RGQ PCR Kit* LoD. Of the 18 samples determined positive by the *therascreen PIK3CA RGQ PCR Kit* and negative by NGS, 11 were low positive (within one ΔC_t of the cutoff using the *therascreen PIK3CA RGQ PCR Kit* and therefore low positive samples). One case was detected as H1047L (3140A>T).

by the *therascreen* PIK3CA RGQ PCR Kit but detected as H1047I (3139_3140CA>AT) by the NGS assay. The underlying cause for the six remaining discordant results was not identified.

Table 14 shows the PPA of the target with NGS as the orthogonal method.

Table 14. Analysis of agreement for FFPE tissue specimens by specific mutation

Mutation*	Positive percent agreement (N)	Two-sided 95% CI
C420R	100.0 (4/4)	39.8, 100.0
E542K	100.0 (27/27)	87.2, 100.0
E545G	100.0 (3/3)	29.2, 100.0
E545K	100.0 (49/49)	92.7, 100.0
E545A	100.0 (2/2)	15.8, 100.0
Q546E	100.0 (1/1)	2.5, 100.0
Q546R	50.0 (1/2)	1.3, 98.7
H1047L	100.0 (12/12)	73.5, 100.0
H1047R	98.1 (101/103)	93.2, 99.8

* All 11 PIK3CA mutations were detected in tissue specimen in the SOLAR-1 trial (Table 15).

Clinical performance: Tissue specimens

The *therascreen PIK3CA RGQ PCR Kit* is intended to be used as a companion diagnostic test to aid clinicians in identifying breast cancer patients who may be eligible for treatment with PIQRAY (alpelisib) based on the presence of one or more *PIK3CA* mutations detected in clinical FFPE breast tumor tissue specimens.

Clinical outcome data

The SOLAR-1 study, CBYL719C2301, was a randomized, double-blind, placebo-controlled, international, multicenter Phase III clinical trial determining the efficacy and safety of treatment with PIQRAY (alpelisib) plus fulvestrant versus placebo plus fulvestrant in men and postmenopausal women with HR+, HER2-negative advanced breast cancer that progressed on or after aromatase inhibitor treatment. A total of 572 breast cancer patients were enrolled into two cohorts, with or without a *PIK3CA* mutation. Patients were randomized to receive either PIQRAY (alpelisib) 300 mg plus fulvestrant or placebo plus fulvestrant in a 1:1 ratio. Randomization was stratified by presence of lung and/or liver metastasis and previous treatment with CDK4/6 inhibitor(s).

The primary endpoint for the study was progression-free survival (PFS) using Response Evaluation Criteria in Solid Tumors (RECIST v1.1), based on investigator assessment in advanced breast cancer patients enrolled with a *PIK3CA* mutation. Other secondary endpoints included PFS for patients without *PIK3CA* mutation, as well as overall survival (OS), overall response rate (ORR) and clinical benefit rate (CBR) by *PIK3CA* cohort (i.e., with or without *PIK3CA* mutation).

PIK3CA mutation status for screening and enrollment of patients was determined centrally by a clinical trial assay (CTA) or the QIAGEN *therascreen PIK3CA RGQ PCR Kit*, testing FFPE breast cancer tumor specimens. Of the 572 patients randomized in SOLAR-1, 177 patients (30.9% of the study population, including 172 *PIK3CA* mutation-positive and five *PIK3CA* mutation-negative patients) were randomized using the *therascreen PIK3CA RGQ PCR Kit*. All other patients (395) were randomized using the CTA (69.1% of the study population, including 169 *PIK3CA* mutation-positive and 226 *PIK3CA* mutation-negative patients).

PIQRAY (alpelisib) in combination with fulvestrant demonstrated superiority over fulvestrant alone for the primary endpoint of PFS per investigator assessment using RECIST 1.1 in the *PIK3CA* mutant cohort. An estimated 35% risk reduction in disease progression or death was observed in favor of the PIQRAY (alpelisib) plus fulvestrant arm, relative to the placebo plus fulvestrant arm [Hazard ratio [HR] = 0.65; 95% CI: 0.50, 0.85; $p = 0.0013$, based on a two-sided stratified log-rank test]. Median PFS was prolonged by a clinically meaningful 5.3 months, from 5.7 months in the placebo plus fulvestrant arm to 11.0 months in the PIQRAY (alpelisib) plus fulvestrant arm (Figure 20).

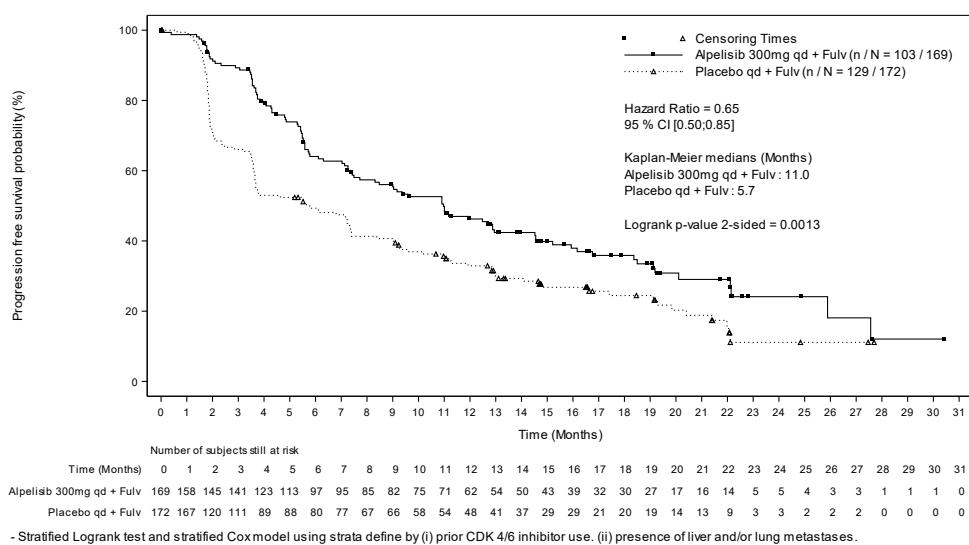


Figure 20. Kaplan-Meier plot of PFS by treatment in the *PIK3CA* mutant patients randomized in SOLAR-1.

Samples from the 395 patients who were randomized using the CTA were retested retrospectively by the *therascreen PIK3CA RGQ PCR Kit* and yielded 389 samples evaluable by *therascreen PIK3CA RGQ PCR Kit* (98.5%) with six patient samples not evaluable by *therascreen PIK3CA RGQ PCR Kit* (Table 16).

Table 15. Prevalence of *PIK3CA* mutations detected by the *therascreen PIK3CA RGQ PCR Kit* in tissue specimens in the SOLAR-1 clinical trial

Exon	Mutation*	COSMIC ID†	Base change	Frequency in FFPE tissue specimens N = 374 (%)
7	C420R	757	1258 T>C	6 (1.6)
9	E542K	760	1624 G>A	66 (17.6)
	E545A	12458	1634 A>C	4 (1.1)
	E545D	765	1635 G>T	6 (1.6)
	E545G	764	1634 A>G	9 (2.4)
	E545K	763	1633 G>A	91 (24.3)
	Q546E	6147	1636 C>G	1 (0.3)
	Q546R	12459	1637 A>G	2 (0.5)
20	H1047L	776	3140 A>T	24 (6.4)
	H1047R	775	3140 A>G	160 (42.8)
	H1047Y	774	3139 C>T	5 (1.3)

* A *PIK3CA* mutation-positive patient may have more than one mutation.

† COSMIC: Catalogue of somatic mutations in cancer: <https://cancer.sanger.ac.uk/cosmic>.

N = number of *PIK3CA* mutation-positive patients identified by FFPE tissue specimen in SOLAR-1.

Table 16. Disposition of retrospective retested (CTA-enrolled) subjects (full analysis set, CTA-enrolled)

<i>therascreen PIK3CA RGQ PCR Kit</i> results	CTA positive (N = 169)	CTA negative (N = 226)	Total (N = 395)
Valid	169 (100.0%)	220 (97.3%)	389 (98.5%)
Positive	164 (97.0%)	11 (4.9%)	175 (44.3%)
Negative	5 (3.0%)	209 (92.5%)	214 (54.2%)
Invalid	0 (0%)	6 (2.7%)	6 (1.5%)

To evaluate concordance between the CTA and the *therascreen PIK3CA RGQ PCR Kit*, the concordance indices PPA, NPA, and OPA, together with the respective two-sided Clopper-Pearson Exact 95% confidence intervals were calculated.

Table 17 shows the *therascreen PIK3CA RGQ PCR Kit* evaluable subset using the CTA as the reference and indicates a high level of agreement between the CTA and *therascreen PIK3CA RGQ PCR Kit* results.

Table 18 uses the *therascreen PIK3CA RGQ PCR Kit* as the reference and indicates a high level of agreement between the CTA and *therascreen PIK3CA RGQ PCR Kit* results.

Table 17. *therascreen PIK3CA RGQ PCR Kit* versus CTA (with CTA as reference)

Measure of agreement	Percent agreement, %	Two-sided 95% CI
Positive percent agreement (PPA)	97.0	93.2, 99.0
Negative percent agreement (NPA)	95.0	91.2, 97.5
Overall percent agreement (OPA)	95.9	93.4, 97.6

Table 18. *therascreen PIK3CA RGQ PCR Kit* versus CTA (with *therascreen PIK3CA RGQ PCR Kit* as reference)

Measure of agreement	Percent agreement, %	Two-sided 95% CI
Positive percent agreement (PPA)	93.7	89.0, 96.8
Negative percent agreement (NPA)	97.7	94.6, 99.2
Overall percent agreement (OPA)	95.9	93.4, 97.6

Table 19 shows the PPA, NPA, and OPA estimates recalculated to adjust for enrichment due to the six missing *therascreen PIK3CA RGQ PCR Kit* results in the CTA mutation-negative patients.

Table 19. *therascreen PIK3CA RGQ PCR Kit* versus CTA (with *therascreen PIK3CA RGQ PCR Kit* as reference)

Measure of agreement	Percent agreement, %	Two-sided 95% CI
Positive percent agreement (PPA)	93.6	90.1, 97.0
Negative percent agreement (NPA)	97.7	95.6, 99.5
Overall percent agreement (OPA)	95.9	93.8, 97.8

The primary PFS analysis for clinical utility of the *therascreen PIK3CA RGQ PCR Kit* demonstrated similar clinical efficacy to that determined in the SOLAR-1 study. Analysis of the *therascreen PIK3CA RGQ PCR Kit* mutant-positive patient subset (347 patients) demonstrated that patients randomized to the PIQRAY (alpelisib) plus fulvestrant arm had an estimated 36% lower risk of disease progression or death (HR = 0.64; 95% CI: 0.48, 0.85) than patients randomized to the placebo plus fulvestrant arm.

Sensitivity analyses assessed the impact of the missing *therascreen PIK3CA RGQ PCR Kit* data on PFS and demonstrated that the results were robust to missing data. For example, when assuming the six missing *therascreen PIK3CA RGQ PCR Kit* results were discordant with the CTA results, those *therascreen PIK3CA RGQ PCR Kit* mutation-positive patients randomized to the PIQRAY (alpelisib) plus fulvestrant arm had an estimated 37% lower risk of disease progression or death (HR = 0.63; 95% CI [0.47, 0.84]) than patients randomized to the placebo plus fulvestrant arm.

All CTA-enrolled mutation-positive patients were *therascreen PIK3CA RGQ PCR Kit*-evaluable and only six CTA-enrolled mutation-negative patients were *therascreen PIK3CA RGQ PCR Kit*-unevaluable. Consequently, there was no bias in results through study sample evaluability.

PFS was also estimated in the *therascreen PIK3CA RGQ PCR Kit*-negative population and no PFS benefit was observed in those patients (HR = 0.85; 95% CI: 0.58, 1.25).

Performance Characteristics: Plasma Specimens

Analytical performance: Plasma specimens

The specific performance characteristics of the *therascreen PIK3CA RGQ PCR Kit* were determined in studies using clinical plasma specimens collected from breast cancer patients, contrived plasma specimens comprising healthy donor (HD) plasma spiked with fragmented cell-line DNA from 11 human cell-line specimens that harbor known *PIK3CA* mutations detected by the assay and one *PIK3CA* wild-type cell line specimen (i.e., no mutations as claimed to be detected by the *therascreen PIK3CA RGQ PCR Kit* in exons 7, 9, and 20).

Limit of blank (LoB): Plasma specimens

The limit of blank (LoB) is defined in CLSI guideline EP17-A2 as “the highest measurement result that is likely to be observed (with a stated probability) for a blank sample”. For the *therascreen PIK3CA RGQ PCR Kit* this is the data point that corresponds to the upper 95% percentile in the blank samples. To assess performance of the *therascreen PIK3CA RGQ PCR Kit* in the absence of template, and to ensure that a sample with wild-type DNA does not generate an analytical signal that may indicate a low concentration of mutation, a total of 60 unique HD specimens spiked with serially diluted fragmented wild-type PIK3CA DNA at six input levels were tested in triplicate in a study following guidance from CLSI guideline EP17-A2 to determine the LoB for each mutation assay. All mutation assays gave LoB values above the cutoff for their respective mutations. The LoB of the *PIK3CA* mutants detected by the *therascreen PIK3CA RGQ PCR Kit* from plasma specimens is shown below (Table 20).

Table 20. Summary of LoB results

Exon	Mutation	Base change	LoB (ΔC_T value)	False positive call rate (%)
7	C420R	1258T>C	11.15	0%
9	E542K	1624G>A	8.32	0%
	E545A	1634A>C	15.82	0%
	E545D	1635G>T	9.13	0%
	E545G	1634A>G	13.39	0%
	E545K	1633G>A	15.74	0%
	Q546E	1636C>G	15.82	0%
	Q546R	1637A>G	10.19	0.56%
	H1047L	3140A>T	15.55	0.56%
20	H1047R	3140A>G	11.93	0%
	H1047Y	3139C>T	9.89	0%

Limit of detection (LoD): Plasma specimens

A study was conducted to determine the LoD of each of the 11 *PIK3CA* mutations using contrived plasma specimens. LoD was defined as the lowest amount of mutant DNA in a background of wild-type DNA at which a mutant sample will provide mutation positive results in 95% of the test results (C_{95}).

To determine the LoD for each mutation, samples with different percentages of mutation were prepared at low DNA input and tested with the *therascreen PIK3CA RGQ PCR Kit* (Table 21). The LoD for each assay was calculated using a “probit” method. The LoD of 11 contrived mutant samples were established using three different *therascreen PIK3CA RGQ PCR Kit* lots with 24 replicates tested per kit lot per level. A subset of the mutations were verified using clinical plasma samples at the determined LoD.

Table 21. LoD for plasma specimens established using low DNA input clinical and contrived plasma specimens

Exon	Mutation	COSMIC* ID	Base change	LoD, % MAF
7	C420R	757	1258T>C	4.46 [†]
9	E542K	760	1624G>A	5.06 ^{†‡}
	E545A	12458	1634A>C	1.82 [†]
	E545D	765	1635G>T	3.21 [†]
	E545G	764	1634A>G	1.94 ^{†‡}
	E545K	763	1633G>A	2.42 ^{†‡}
	Q546E	6147	1636C>G	5.31 [†]
	Q546R	12459	1637A>G	4.22 [†]
20	H1047L	776	3140A>T	2.37 ^{†‡}
	H1047R	775	3140A>G	1.98 ^{†‡}
	H1047Y	774	3139C>T	7.07 [†]

MAF: Mutant allele frequency.

* COSMIC: Catalogue of somatic mutations in cancer: <https://cancer.sanger.ac.uk/cosmic>.

† LoD values were established using cell-line specimens.

‡ LoD values were verified using clinical plasma specimens.

Genomic DNA input range: Plasma specimens

The control C_T working range was set using calculated tolerance intervals and LoB values. The control assay C_T working range was determined using a total of 30 individual 10 ml wild-type samples containing different wild-type DNA concentrations (120 observations). The final control assay C_T working range was set at a C_T value of 24.69 to 31.68 giving a 98% confidence level for 95% of the intended use population.

ΔC_T cutoff values: Plasma specimens

Contrived plasma specimens were used to establish the cutoff values of each mutation. In addition to statistical analysis of ΔC_T values, LoB values and design requirements for false-positive and false-negative rates were used to define acceptable cutoff values.

The cutoff values established are shown in Table 22.

Table 22. Established cutoff values for each mutation assay when testing DNA from plasma specimens

Assay	Cutoff value (ΔC_I)
C420R	≤ 6.0
E542K	≤ 4.8
E545A	≤ 10.0
E545D	≤ 7.0
E545G	≤ 9.5
E545K	≤ 10.0
Q546E	≤ 10.0
Q546R	≤ 7.0
H1047L	≤ 10.0
H1047R	≤ 9.0
H1047Y	≤ 6.2

Effect of DNA input on ΔC_T values (linearity): Plasma specimens

The DNA input level is defined as the total quantity of amplifiable DNA in a sample as determined by the C_T values from the *PIK3CA* control reaction. To demonstrate that the performance of the *therascreen PIK3CA RGQ PCR Kit* is consistent across the control reaction C_T range (24.69 to 31.68), an 8-level serial dilution for each of the 11 *PIK3CA* mutation assays was prepared (fragmented DNA extracted from cell-line specimens). The target C_T values for dilutions levels 1 and 8, for each mutation, were targeted to be above and below the control reaction C_T range. Overall, the ΔC_T values at different total DNA input levels were consistent across the working range of the *therascreen PIK3CA RGQ PCR Kit* for mutations.

Assay specificity (cross-reactivity/specificity): Plasma specimens

To assess whether cross-reactivity between mutations detected by the assay has been correctly accounted for in the setting of the analytical cutoff values, mutant-positive contrived plasma specimens, at high and low DNA input, were diluted to high and low MAF targets and tested in duplicate using three lots of the *therascreen PIK3CA RGQ PCR Kit*. Cross-reactivity was observed between the H1047L and H1047R assays. However, it was determined that this cross-reactivity is unidirectional (i.e., if a double H1047R and H1047L sample is seen, this will only be reported as "H1047R Mutation Detected"). This rule is incorporated in to the automated "therascreen_PIK3CA_Plasma Assay Profile" algorithm.

Interference: Plasma specimens

Endogenous substances

Potential endogenous interfering substances that may be present in the plasma specimens were tested in mutant and wild-type contrived samples at concentrations based on CLSI guideline EP7-A2:

- Hemoglobin (2 g/l)
- Triglycerides (37 mmol/l)
- EDTA (3.4 µmol/l)
- Caffeine (308 µmol/l)
- Albumin (30 mg/ml)
- Conjugated bilirubin (342 µmol/l)
- Unconjugated Bilirubin (342 µmol/l)

The results demonstrated that these substances did not interfere with the results of the *therascreen PIK3CA RGQ PCR Kit*.

Exogenous substances

Potential exogenous interfering substances present in the DNA extraction process were tested in mutant and wild-type samples at concentrations assuming 10% carryover from the extraction process:

- Ethanol
- Proteinase K
- Buffer ACL
- Buffer ACB
- Buffer ACW1
- Buffer ACW2

The results demonstrated that these substances did not interfere with the results of the *therascreen PIK3CA RGQ PCR Kit*.

Lot interchangeability: Plasma specimens

The *therascreen PIK3CA RGQ PCR System* utilizes the QIAamp DSP Circulating Nucleic Acid Kit for extraction of DNA, and the *therascreen PIK3CA RGQ PCR Kit* for the amplification of DNA and detection of *PIK3CA* mutation status. Lot-to-lot reproducibility and interchangeability were demonstrated using three lots of the QIAamp DSP Circulating Nucleic Acid Kit and one lot of the *therascreen PIK3CA RGQ PCR Kit*. The overall percentage of correct calls across lots for all mutation positive and wild-type samples was 100%.

Specimen handling: Plasma specimens

To demonstrate that different laboratories will produce acceptable results when starting from the same plasma specimen, extractions were performed across three different sites. Contrived specimens were used for all 11 mutations, as well as a *PIK3CA* wild-type clinical plasma specimen. 18 x 2 ml aliquots of each specimen were prepared; these aliquots were randomized and split into 18 extract sets. These extract sets were then distributed evenly across the three testing sites (one internal QIAGEN site in the United Kingdom and two additional external sites in the USA); six extracts per study site. Testing of the DNA extracted from the specimen aliquots using the *therascreen PIK3CA RGQ PCR Kit* was performed at the internal QIAGEN site. When comparing the results from each specimen across all three sites, the percentage of correct mutation calls for *PIK3CA* mutation-positive and wild-type specimens was 100%.

Repeatability and reproducibility: Plasma specimens

The repeatability of the *therascreen* PIK3CA RGQ PCR Kit was investigated by testing DNA extracted from cell-line specimens, representing all 11 mutations detected by the *therascreen* PIK3CA RGQ PCR Kit at 1x LoD and 3x LoD.

Repeatability was assessed by testing these samples at one site across 20 non-consecutive days, using three Rotor-Gene Q instruments and by three operators to generate a total of 120 replicates per sample (Table 23).

Table 23. Assay repeatability – proportion of correct calls for PIK3CA mutations tested in DNA samples obtained from plasma specimens

Exon	Mutation	Mutation level	Fractional proportion of valid results	Correct calls, %	Lower two-sided 95% CI
NA	Wild type	C;30	114/120	95.00	89.43
7	C420R	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
9	E542K	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
	E542A	LoD	119/120	99.17	95.44
		3x LoD	120/120	100.00	96.97
11	E545D	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
	E545G	LoD	119/120	99.17	95.44
		3x LoD	120/120	100.00	96.97
13	E545K	LoD*	111/120	92.50	86.24
		3x LoD*	120/120	100.00	96.97
	Q546E	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
15	Q546R	LoD*	115/120	95.83	90.54
		3x LoD*	120/120	100.00	96.97
	H1047L	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97
17	H1047R	LoD	110/120	91.67	85.21
		3x LoD	120/120	100.00	96.97
	H1047Y	LoD	120/120	100.00	96.97
		3x LoD	120/120	100.00	96.97

* For E545K and H1047R the LoD used were 1.99 and 1.44, respectively. The LoD was readjusted and confirmed in a subsequent study. The readjusted LoD was used in the subsequent study (Table 24).

Reproducibility was measured by testing contrived samples at 1x LoD and 3x LoD level samples across three different sites (one internal QIAGEN site in the United Kingdom and two additional external sites in the USA). All of these samples were tested at each site across 10 non-consecutive days, using three Rotor-Gene Q instruments and by three operators to generate a total of 60 replicates per sample (Table 24).

Table 24. Assay reproducibility – proportion of correct calls for PIK3CA mutations tested in DNA samples obtained from plasma specimens across all sites

Exon	Mutation	Mutation level	Fractional proportion of valid result	Correct calls, %	Lower two-sided 95% CI
NA	WT	C<30	223/238	93.70	89.82
7	C420R	LoD	237/238	99.58	97.68
		3x LoD	238/238	100.00	98.46
9	E542K	LoD	237/240	98.75	96.39
		3x LoD	240/240	100.00	98.47
	E545A	LoD	239/240	99.58	97.70
		3x LoD	240/240	100.00	98.47
	E545D	LoD	240/240	100.00	98.47
		3x LoD	240/240	100.00	98.47
	E545G	LoD	237/240	98.75	96.39
		3x LoD	239/239	100.00	98.47
	E545K	LoD*	432/432	100.00	99.15
		3x LoD	240/240	100.00	89.47
	Q546E	LoD	238/238	100.00	98.46
		3x LoD	238/238	100.00	98.46
	Q546R	LoD*	232/240	96.67	93.54
		3x LoD	240/240	100.00	98.47
20	H1047L	LoD	236/238	99.16	97.00
		3x LoD	238/238	100.00	98.46
	H1047R	LoD	430/432	99.54	98.34
		3x LoD	236/236	100.00	98.45
	H1047Y	LoD	239/240	99.58	97.70
		3x LoD	240/240	100.00	98.47

* Samples at revised LoD with E545K and H1047R (as per Table 21) were evaluated for six days across three sites, by three operators, with two runs and four replicates for a total of 144 measurements per site, 432 total across all three sites. Table 25 shows the positive percentage agreement (PPA) of the target with NGS as the orthogonal method.

A variance component analysis was used to estimate the standard deviation for between-kit, between-run, between-operator, between-instrument, between-day and within-run variability for repeatability and reproducibility. Across all variance components, the total standard deviation (SD) was $\leq 1.34 \Delta C_T$ for LoD and $\leq 0.73 \Delta C_T$ for 3x LoD for all PIK3CA mutations tested in the reproducibility testing. Across all mutant panel members, the SD was $\leq 0.20 \Delta C_T$ for LoD and $\leq 0.10 \Delta C_T$ for 3x LoD for between-lots (lot interchangeability). The SD for within-run variability (repeatability/precision) ranged from $0.415 \Delta C_T$ to $1.407 \Delta C_T$ for LoD and $0.206 \Delta C_T$ to $0.583 \Delta C_T$ for 3x LoD.

Blood collection tube validation

The impact of blood to plasma separation time on plasma specimen quality and subsequent results was determined using contrived blood samples for H1047R (the most prevalent mutation) and whole blood specimens from healthy volunteers were used as wild-type specimens. Blood specimens were collected into 10 ml K₂EDTA tubes from four donors (eight tubes per donor). Contrived blood specimens were generated by spiking PIK3CA H1047R mutant fragmented cell-line DNA into blood tubes from two donors after collection. Blood specimens were separated into plasma at approximately 1-, 2-, 3-, and 4-hour time points. DNA was extracted from the plasma specimens using the QIAamp DSP Circulating Nucleic Acid Kit and each target was tested using the *therascreen* PIK3CA RGQ PCR Kit in 16 replicates.

All tested samples were correctly called at each of the time points. In addition, there was no statistically significant drift in ΔC_T observed for the PIK3CA H1047R mutant sample.

This study demonstrated that there is no impact of the blood to plasma separation time, if processed within four hours, on the *therascreen* PIK3CA RGQ PCR Kit.

Accuracy: Comparison to the analytical reference method (plasma specimens)

To demonstrate the accuracy of the *therascreen* PIK3CA RGQ PCR Kit, a study was conducted with specimens from the SOLAR-1 clinical trial relative to a validated NGS assay. The *therascreen* PIK3CA RGQ PCR Kit and NGS testing for *PIK3CA* alterations was performed using the DNA from 552 clinical plasma specimens from the SOLAR-1 clinical trial.

DNA samples with both NGS and *therascreen* PIK3CA RGQ PCR Kit valid results (542/552 samples) were analyzed to assess the positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA). These percentages, together with the corresponding two-sided 95% confidence intervals (CI), are summarized in Table 25.

Table 25. Analysis of agreement for DNA samples obtained from plasma specimens

Measure	Percent agreement (N)	Lower 95% CI
Positive percent agreement	97.39 (149/153)	93.44
Negative percent agreement	91.26 (355/389)	88.00
Overall percent agreement	92.99 (504/542)	90.50

For the 38 discordant overall percentage agreement results:

- Four samples (0.7%) were Wild-Type (i.e., No Mutation Detected) by the *therascreen* PIK3CA RGQ PCR Kit, but Mutation Detected by NGS.
- 34 samples (6.3%) were Mutation Detected by the *therascreen* PIK3CA RGQ PCR Kit, but Wild-Type by NGS.
- Table 26 shows the PPA of the target with NGS as the orthogonal method.

Table 26. Analysis of agreement for DNA samples obtained from plasma specimens by mutation

Mutation*	Positive percent agreement (N)	Two-sided 95% CI
C420R	100.0% (2/2)	15.8,100.0
E542K	90.9% (20/22)	70.8, 98.9
E545G	100.0% (2/2)	15.8,100.0
E545K	100.0% (38/38)	90.7,100.0
H1047L	100.0% (5/5)	47.8,100.0
H1047R	97.6% (83/85)	91.8, 99.7

* 6/11 *PIK3CA* mutations were detected by plasma specimen in the SOLAR-1 trial (Table 31).

Clinical performance: Plasma specimens

The *therascreen PIK3CA RGQ PCR Kit* is intended to be used as a companion diagnostic test to aid clinicians in identifying breast cancer patients who may be eligible for treatment with PIQRAY (alpelisib) based on the presence of one or more *PIK3CA* mutations detected in K₂EDTA anticoagulated peripheral venous whole blood clinical plasma specimens.

K₂EDTA anticoagulated peripheral venous whole blood clinical plasma specimens collected from breast cancer patients randomized in SOLAR-1 prior to initiation of study treatment (baseline) were tested retrospectively with the *therascreen PIK3CA RGQ PCR Kit* to evaluate the clinical utility of this specimen type for determination of *PIK3CA* mutation status and to evaluate concordance between tissue and plasma results.

Concordance analysis results

Concordance of the *therascreen PIK3CA RGQ PCR Kit* using plasma results to the *therascreen PIK3CA RGQ PCR Kit* using tissue results is shown in Table 27. Of the 328 *therascreen PIK3CA RGQ PCR Kit* tissue-positive patients, 179 were *therascreen PIK3CA RGQ PCR Kit* plasma-positive. Of the 215 *therascreen PIK3CA RGQ PCR Kit* tissue-negative patients, 209 were *therascreen PIK3CA RGQ PCR Kit* plasma-negative. There were no invalid plasma results.

Table 27. Correspondence table between *therascreen PIK3CA RGQ PCR Kit* tissue results and *therascreen PIK3CA RGQ PCR Kit* plasma results

		<i>therascreen PIK3CA RGQ PCR Kit</i> tissue		
<i>therascreen PIK3CA RGQ PCR Kit</i> plasma	Positive	Negative	Invalid	Total
Positive	179	6	1	186
Negative	149	209	5	363
Invalid	0	0	0	0
Total	328	215	6	549

Agreement (PPA, NPA and OPA) between the *therascreen PIK3CA RGQ PCR Kit* plasma and *therascreen PIK3CA RGQ PCR Kit* tissue results was calculated using the *therascreen PIK3CA RGQ PCR Kit* tissue results as reference (Table 28). The point estimates of PPA, NPA, and OPA were 55%, 97%, and 72%, respectively.

Table 28. Agreement between *therascreen PIK3CA RGQ PCR Kit* plasma results and *therascreen PIK3CA RGQ PCR Kit* tissue results using the *therascreen PIK3CA RGQ PCR Kit* tissue results as reference

Measure of agreement	Percent agreement (N)	95% CI*
Positive percent agreement	55% (179/328)	(49.0, 60.1)
Negative percent agreement	97% (209/215)	(94.0, 99.0)
Overall percent agreement	72% (388/543)	(67.5, 75.2)

* 95% CI calculated using the Clopper-Pearson Exact method.

Confirmatory testing of the plasma samples with a validated reference NGS test method confirmed 91% of the *therascreen PIK3CA RGQ PCR Kit* plasma results. Of the *therascreen PIK3CA RGQ PCR Kit* tissue-positive patients that were *therascreen PIK3CA RGQ PCR Kit* plasma-negative, NGS confirmed the *therascreen PIK3CA RGQ PCR Kit* plasma-negative results in 80% of the cases. Of the six discordant *therascreen PIK3CA RGQ PCR Kit* plasma-positive, *therascreen PIK3CA RGQ PCR Kit* tissue-negative patients, five were confirmed plasma-positive by NGS.

Progression-free survival (PFS) analysis

PFS for PIQRAY (alpelisib) in combination with fulvestrant for the *therascreen PIK3CA RGQ PCR Kit* plasma-positive population ($N = 185$) was observed in the favor of the PIQRAY (alpelisib) plus fulvestrant arm compared to the placebo plus fulvestrant arm with an estimated 46% risk reduction in disease progression or death (HR = 0.54, 95% CI: 0.33, 0.88) (Table 29). In comparison, the PFS HR in the *therascreen PIK3CA RGQ PCR Kit* tissue-positive population was 0.64 (95% CI: 0.48, 0.85) and 0.65 (95% CI: 0.50, 0.85) in the SOLAR-1 *PIK3CA* mutant cohort as determined by the enrolling tissue assay.

Table 29. PFS analysis in the *therascreen PIK3CA RGQ PCR Kit* plasma-positive patients

PFS (N)	HR (95% CI) PIQRAY 300 mg qd + fulv/placebo qd + fulv*
<i>therascreen PIK3CA RGQ PCR Kit</i> plasma-positive (185)	0.54 (0.33, 0.88)

* HR and 95% CI calculated using enrichment adjustment.

The PFS HR for the 179 *therascreen PIK3CA RGQ PCR Kit* tissue-positive *therascreen PIK3CA RGQ PCR Kit* plasma-positive patients was 0.53 (95% CI: 0.33, 0.84). Median PFS was 10.9 months for the PIQRAY (alpelisib) plus fulvestrant arm versus 3.6 months for the placebo plus fulvestrant arm (Table 30, Figure 21).

Table 30. Progression-free survival (months) in the *therascreen PIK3CA RGQ PCR Kit* tissue-positive, *therascreen PIK3CA RGQ PCR Kit* plasma-positive patients

Progression-free survival	PIQRAY 300 mg qd + fulv N=90	Placebo qd + fulv N=89	HR (95% CI) PIQRAY 300 mg qd + fulv/placebo qd + fulv
No of events (%)	57 (63.3)	72 (80.9)	0.53 (0.33, 0.84)
PD (%)	55 (61.1)	67 (75.3)	-
Death (%)	2 (2.2)	5 (5.6)	-
No. censored (%)	33 (36.7)	17 (19.1)	-
Median (95% CI)	10.9 [7.0, 16.2]	3.6 [2.0, 5.8]	-

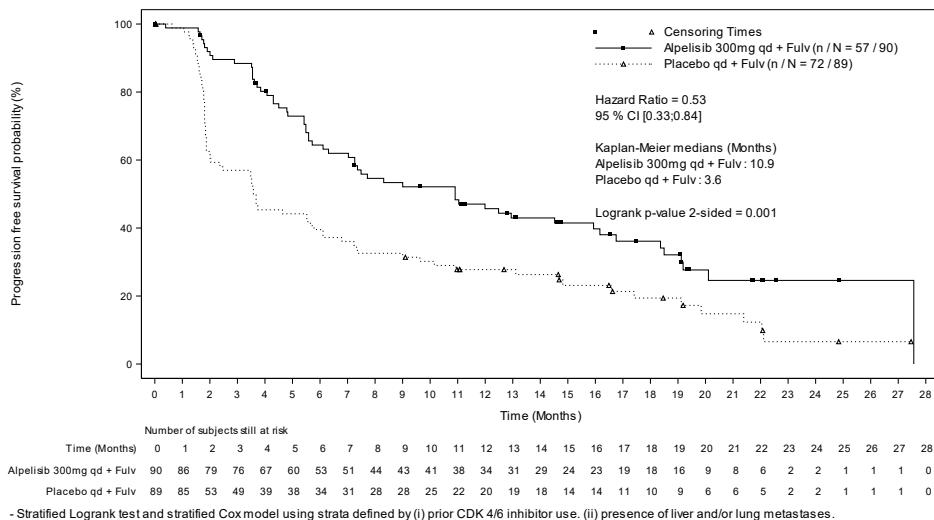


Figure 21. Kaplan-Meier plot of PFS by treatment in *therascreen PIK3CA RGQ PCR Kit* tissue-positive *therascreen PIK3CA RGQ PCR Kit* plasma-positive patients.

Table 31. Prevalence of *PIK3CA* mutations detected by the *therascreen* PIK3CA RGQ PCR Kit in plasma specimens in the SOLAR-1 clinical trial

Exon	Mutation*	COSMIC ID [†]	Base change	Frequency in plasma specimens N = 186 (%)
7	C420R	757	1258 T>C	2 (1.1)
9	E542K	760	1624 G>A	22 (11.8)
	E545A	12458	1634 A>C	0 (0.0)
	E545D	765	1635 G>T	0 (0.0)
	E545G	764	1634 A>G	3 (1.6)
	E545K	763	1633 G>A	48 (25.8)
	Q546E	6147	1636 C>G	0 (0.0)
	Q546R	12459	1637 A>G	0 (0.0)
20	H1047L	776	3140 A>T	10 (5.4)
	H1047R	775	3140 A>G	102 (54.8)
	H1047Y	774	3139 C>T	0 (0.0)

* A *PIK3CA* mutation-positive patient may have more than one mutation.

† COSMIC: Catalogue of somatic mutations in cancer: <https://cancer.sanger.ac.uk/cosmic>.

N = number of *PIK3CA* mutation-positive patients identified by plasma specimen in SOLAR-1.

Safety and effectiveness conclusions

The clinical accuracy study met the acceptance criteria for PPA for mutation-positive samples and NPA for mutation-negative samples therefore confirming that the plasma *therascreen PIK3CA RGQ PCR Kit* generated accurate results for both biomarker-positive and -negative intended use samples.

Concordance of the plasma *therascreen PIK3CA RGQ PCR Kit* with the tissue *therascreen PIK3CA RGQ PCR Kit* results for NPA was 97% and demonstrated a low risk of false-positives. A false-negative result may prevent a patient from accessing a potentially beneficial drug. There was 55% PPA for plasma/tissue that indicates that plasma-negative patients may be *PIK3CA* mutation-positive by tissue. Therefore, where patients' plasma showed *PIK3CA* mutation-negative results with the *therascreen PIK3CA RGQ PCR Kit*, a tissue specimen should be tested for confirmation of *PIK3CA* mutation status.

Clinical efficacy of PIQRAY (alpelisib) in combination with fulvestrant for the *therascreen PIK3CA RGQ PCR Kit* plasma *PIK3CA* mutation-positive population, as identified by the *therascreen PIK3CA RGQ PCR Kit*, was demonstrated with an estimated 46% risk reduction in disease progression or death compared to placebo plus fulvestrant (HR = 0.54, 95% CI: 0.33, 0.88).

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

"No C_T value" flag in Positive Control (PC)

- | | |
|--|---|
| a) Incorrect configuration of the PCR | Check your pipetting scheme and repeat the PCR. |
| b) The storage conditions for one or more kit components did not comply with the instructions given in "Reagent Storage and Handling", page 22 | Check the storage conditions (see the kit label) of the reagents and use a new kit, if necessary. |

"Unexpected C_T value" flag in NTC

- | | |
|--|--|
| Contamination occurred during preparation of the PCR | Ensure the area has been decontaminated. Repeat the PCR with new reagents. If possible, close the PCR tubes directly after addition of the sample to be tested. Make sure that work space and instruments are decontaminated at regular intervals. |
|--|--|

"Above acceptable range" or "Below acceptable range" flag in PC

- | | |
|-------------------------------------|---|
| Error during preparation of the PCR | Repeat the PCR ensuring accurate pipetting. |
|-------------------------------------|---|

"DNA input too high" flag in the sample tube

- | | |
|----------------------------|--|
| Sample is too concentrated | Dilute sample to increase C _T value. Samples should be diluted using the water provided in the kit (Water for Dilution [Dil.]). |
|----------------------------|--|

Comments and suggestions

"Above acceptable range" flag in sample tube

Insufficient starting DNA template present in sample

Tissue specimens: Retest one more time. If system shows the same flag second time, re-extract DNA using two slides from the same specimen of resected tissue and an adequate number of slides for CNBs to obtain 20 mm² and repeat PCR. If, after re-extraction, the system shows the same flag for the sample, retest for a second time. If the flag occurs again, the sample is not suitable for use. It should be recorded as "indeterminate" and no further testing carried out.

Plasma specimens: Retest one more time. If system shows the same flag second time, re-extract DNA using 2 ml of patient plasma. If, after re-extraction, the system shows the same flag for the sample, the sample is not suitable for use, it must be recorded as "indeterminate" and no further testing carried out. Consider repeating testing with a fresh specimen of blood plasma.

"IC above acceptable range" flag in the sample tube

Error during preparation of the PCR or inhibitor present in reaction

Tissue specimens: Retest one more time. If system shows the same flag a second time, re-extract DNA using two slides from the same specimen of resected tissue or an adequate number of slides for CNBs to obtain 20 mm² and repeat PCR. If, after re-extraction, the system shows the same flag for the sample, retest for a second time. If the flag occurs again, the sample is not suitable for use. It should be reported as "indeterminate" and no further testing carried out.

Plasma specimens: Retest one more time. If system shows the same flag a second time, re-extract DNA using 2 ml of patient plasma. If, after re-extraction, the system shows the same flag for the sample, the sample is not suitable for use, it must be recorded as "indeterminate" and no further testing carried out. Consider repeating testing with a fresh specimen of blood plasma.

"No C_T value" flag in T1 Control (sample)

No amplifiable DNA template present in sample

Tissue specimens: Retest one more time. If system shows the same flag second time, re-extract DNA using two slides from the same specimen of resected tissue or an adequate number of slides for CNBs to obtain 20 mm² and repeat PCR. If, after re-extraction, the system shows the same flag for the sample, retest for a second time. If the flag occurs again, the sample is not suitable for use. It should be recorded as "indeterminate" and no further testing carried out.

Plasma specimens: Retest one more time. If system shows the same flag second time, re-extract DNA using 2 ml of patient plasma. If, after re-extraction, the system shows the same flag for the sample, the sample is not suitable for use, it must be recorded as "indeterminate" and no further testing carried out. Consider repeating testing with a fresh specimen of blood plasma.

References

1. Katso, R., Okkenhaug, K., Ahmadi, K., et al. (2001) Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell. Dev. Biol.* **17**, 615.
2. Samuels, Y., Wang, Z., Bardelli, A., et al. (2004) High frequency of mutations of the PIK3CA gene in human cancers. *Science*. **304**, 554.
3. Cancer Genome Atlas Network (2012). Comprehensive molecular portraits of human breast tumors. *Nature*. **490**, 61.
4. National Breast Cancer Foundation (2018). Breast cancer facts. Available at: www.nationalbreastcancer.org/breast-cancer-facts. Accessed: 14 January 2019.
5. Siegel, R.L., Miller, K.D., Jemal, A. (2018). Cancer statistics, 2018. *CA Cancer J. Clin.* **68**, 7.
6. Malvezzi, M., Carioli, G., Bertuccio, P., et al. (2018). European cancer mortality predictions for the year 2018 with focus on colorectal cancer. *Ann. Oncol.* **29**, 1016.

Contact Information

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

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
	Marking for European conformity
	Contains reagents sufficient for <N> reactions
	Use by
	in vitro diagnostic medical device
	Catalog number
	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
	Temperature limitation

Symbol	Symbol definition
	Manufacturer
	Consult instructions for use
	Caution

Ordering Information

Product	Contents	Cat. no.
therascreen PIK3CA RGQ PCR Kit (24)	For 24 reactions: 6 Reaction Mixes, Positive Control, <i>Taq</i> DNA Polymerase, Water for NTC, and Water for Sample Dilution	873111
QIAamp DSP DNA FFPE Tissue Kit		
QIAamp DSP DNA FFPE Tissue Kit (50)	For 50 DNA preps: QIAamp MinElute® columns, Proteinase K, Buffers, and Collection Tubes (2 ml)	60404
QIAamp DSP Circulating Nucleic Acid Kit		
QIAamp DSP Circulating Nucleic Acid Kit (50)	For 50 DNA preps: QIAamp MinElute columns, Proteinase K, Buffers, and Collection Tubes (2 ml)	61504
Rotor-Gene Q MDx 5plex HRM and accessories		
Rotor-Gene Q MDx 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9002032
Rotor-Gene Q MDx 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9002033

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
QIAvac 24 Plus vacuum manifold	For ctDNA purification	19413
QIAvac Connecting System	For ctDNA purification	19419
Vacuum Pump	For ctDNA purification; Or equivalent pump capable of producing a vacuum of –800 to –900 mbar	84010

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

Document Revision History

Date	Changes
R1, June 2019	Initial release
R2, September 2019	Correction on Frequency column in Table 15; Fixed typographical errors; Layout updates

Page left intentionally blank

Page left intentionally blank

Limited License Agreement for *therascreen* PIK3CA RGQ PCR Kit

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

1. The product may be used solely in accordance with the protocols provided with the product and this handbook and for use with components contained in the panel only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this panel with any components not included within this panel except as described in the protocols provided with the product, this handbook, and additional protocols available at www.qiagen.com. Some of these additional protocols have been provided by QIAGEN users for QIAGEN users. These protocols have not been thoroughly tested or optimized by QIAGEN. QIAGEN neither guarantees them nor warrants that they do not infringe the rights of third-parties.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this panel and/or its use(s) do not infringe the rights of third-parties.
3. This panel and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the panel agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

Notice to Purchaser: The purchase of this product conveys to the purchaser the limited, non-transferable right to use only this amount of the product to practice the patented peptide nucleic acid (PNA) process solely for activities of the purchase as set forth in the accompanying QIAGEN instruction manual or package insert, within the field of human diagnostics. By purchasing this product, the purchaser agrees not to: (1) resell the product in any form; (2) use the product for applications in forensics; or (3) use the product for purposes other than what is indicated in this Limited Use Label License. Further information about acquiring rights under patents held by Applied Biosystems LLC may be obtained by contacting the Licensing Department, Thermo Fisher Scientific, 5791 Van Allen Way, Carlsbad CA 92008: phone (760) 603-7200: email outlicensing@lifetech.com.

For updated license terms and product-specific disclaimers, see www.qiagen.com.

Trademarks: QIAGEN®, Sample to Insight®, QIAamp®, MinElute®, Rotor-Gene®, Rotor-Gene AssayManager®, *therascreen*® (QIAGEN Group); DNAZap™ (Thermo Fisher Scientific, Inc.); PIGRAY® (Novartis AG). Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

1116336 Sep-19 HB-2635-001 © 2019 QIAGEN, all rights reserved.

Ordering www.qiagen.com/shop | Technical Support support.qiagen.com | Website www.qiagen.com