

therascreen® PIK3CA RGQ PCR Kit

Instructions for Use



Version 1

IVD

For in vitro diagnostic use

Rx ONLY

(For prescription use only)

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

For use with QIAamp® DSP DNA FFPE Tissue Kit

For use with QIAamp® Circulating Nucleic Acid Kit

REF

873121



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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 test is intended to aid clinicians in identifying 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 with FFPE tumor tissue 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 (US) instrument is used for automated amplification and detection. The kit is to be used by trained personnel in a professional laboratory environment.

Limitations of the Procedure

- Results from the product must be interpreted within the context of all relevant clinical and laboratory findings and are not to be used alone for diagnosis.
- 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 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.
- When testing high MAF samples, it is possible in rare instances that multiple mutation calls may be observed due to cross-reactivity between the target mutations. In these instances, the mutation call with the lowest Delta Ct value is the correct mutation call.

- 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 (US) instruments.
- The product is intended for use only on a Rotor-Gene Q MDx (US) real-time PCR cyclers. 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 is required for optimal results. Dilution of the reagents is not recommended and will result in a loss of performance.
- This Instructions for Use (IFU) is intended to be used with Rotor-Gene AssayManager[®] version 2.1 software 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 in the United States after skin cancers and the second leading cause of cancer-related death (4). In 2023, it was estimated that 297,790 women would be diagnosed with breast cancer in the United States (representing approximately 31% of all cancers in women) and that 43,170 deaths would be recorded (5). In Europe, 99,584 women were predicted to die from breast cancer in 2023 (6). Breast cancer in men is rare, with <1% of breast cancer diagnoses in male patients (5); 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 (US) 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 6 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 (US) 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).

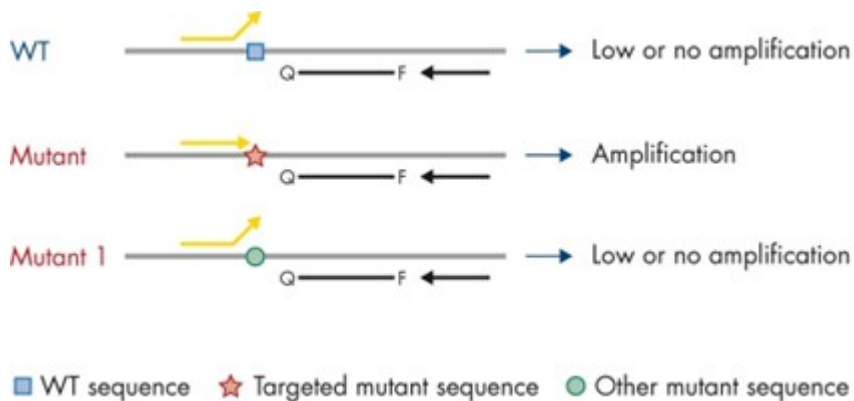


Figure 1. Identification of specific mutation by ARMS PCR. WT: Wild type. Q—F: Double-dye probe. ⇄: Forward and reverse primers.

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 emission.

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).

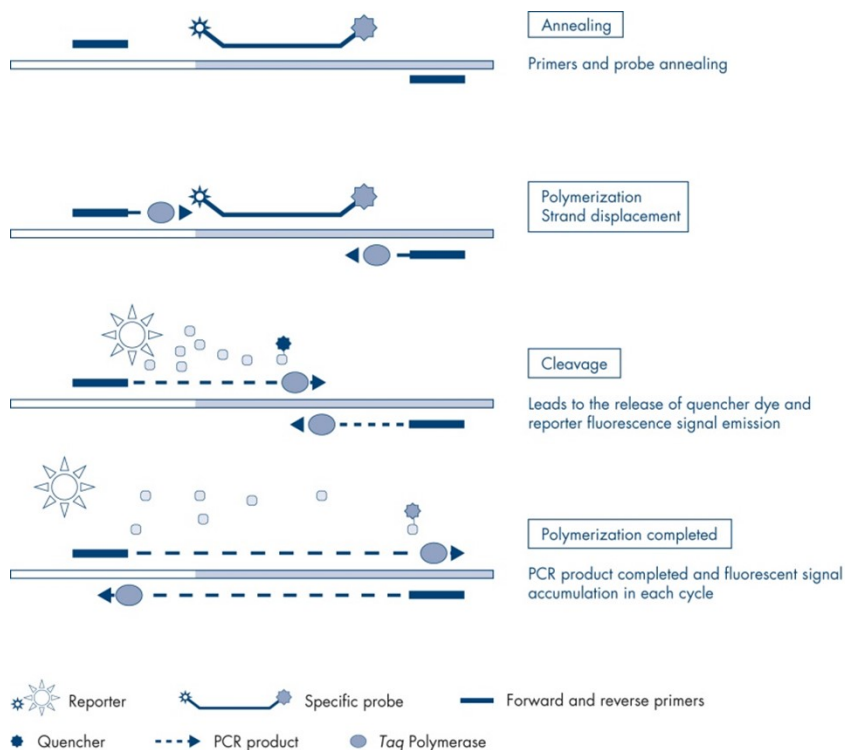
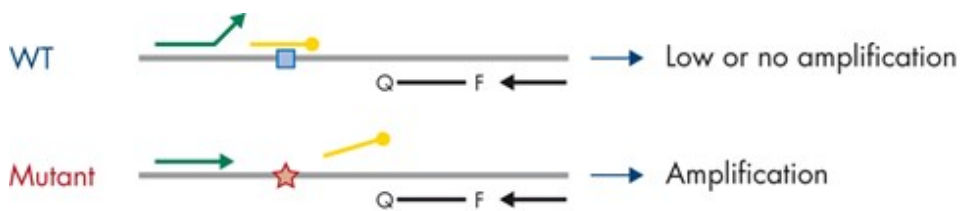


Figure 2. Reaction principle with hydrolysis probes.

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).



■ WT sequence ★ Targeted mutant sequence
● 3'-phosphate oligonucleotide (CLAMP)

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 5 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 (US) instrument operating with a personal computer installed with:

- Rotor-Gene AssayManager version 2.1
- Gamma MDx Plug-in version 1.0.0
- *therascreen*_PIK3CA_FFPE_ MDx Assay Profile version 1.0.1 for analysis of tissue specimens
- *therascreen*_PIK3CA_Plasma_ MDx Assay Profile version 1.0.2 for analysis of plasma specimens

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

Refer to the *Rotor-Gene AssayManager version 2.1 MDx Core Application User Manual* and the *Rotor-Gene AssayManager version 2.1 Gamma MDx (US) Plug-in User Manual* for further information concerning the software.

Run parameters

The Rotor- Gene Q MDx (US) 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</i> PIK3CA RGQ PCR Kit		
Catalog no.	873121	
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
<i>Taq</i> 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

Materials Required but Not Provided

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 PDF at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

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" on page 29) or QIAamp DSP Circulating Nucleic Acid Kit (QIAGEN, cat. no. 61504, see "DNA extraction from plasma specimens" on page 30)
- PCR-degrading solutions (DNAZAP™ or equivalent)
- High Level Laboratory Disinfectant (Distel or equivalent) 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 Q MDx (US) instrument with 72-well rotor (QIAGEN, cat. no. 9002035)
- Rotor-Gene AssayManager version 2.1 Gamma MDx Plug-in and therascreen_PIK3CA_FFPE_ MDx Assay Profile version 1.0.1 and/or therascreen_PIK3CA_Plasma_ MDx Assay Profile version 1.0.2 Assay Profile
- Loading Block 72 × 0.1 mL Tubes, aluminum block for manual reaction set up (QIAGEN, cat. no. 9018901)
- 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

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

Warnings and Precautions

For in vitro diagnostic use.

For prescription use only.

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 PDF 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 (US) instrument.

For safety information regarding the Rotor-Gene Q MDx (US) 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 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 Kit, 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 (US) 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 PCR-degrading solutions such as DNAZap followed by High Level Laboratory Disinfectant such as Distel 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 (US) instrument until the run has finished.
- Do not open Rotor-Gene Q tubes after the run has finished.
- Caution must be observed to ensure correct sample testing to avoid wrong sample entry, loading error. and pipetting error.

Taq DNA Polymerase



Contains: Ethoxylated nonylphenol. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear protective gloves/ protective clothing/ eye protection/ face protection. Dispose of contents/ container to an approved facility in accordance with local, regional, national and international regulations.

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, the outer packaging has been opened during transit, or the shipment does not contain a packing note or the reagents, please contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Storage conditions

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 until the stated expiry date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 6 freeze-thaw cycles.

The reagents must be thawed at room temperature (15 – 25°C) for a minimum of 1 hour and a maximum of 4.5 hours. Once the reagents are ready to use, the PCR reactions can be set up. The Rotor-Gene Q tubes, containing the master mixes and the DNA sample, should be loaded onto Rotor-Gene Q MDx (US) immediately. The total time prior to setup should not exceed 7.5 hours if stored at ambient temperature. Please note that 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.

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

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 μ m 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 2 separate workflows when using FFPE tumor tissue resected specimens and FFPE CNB specimens (Figure 4).

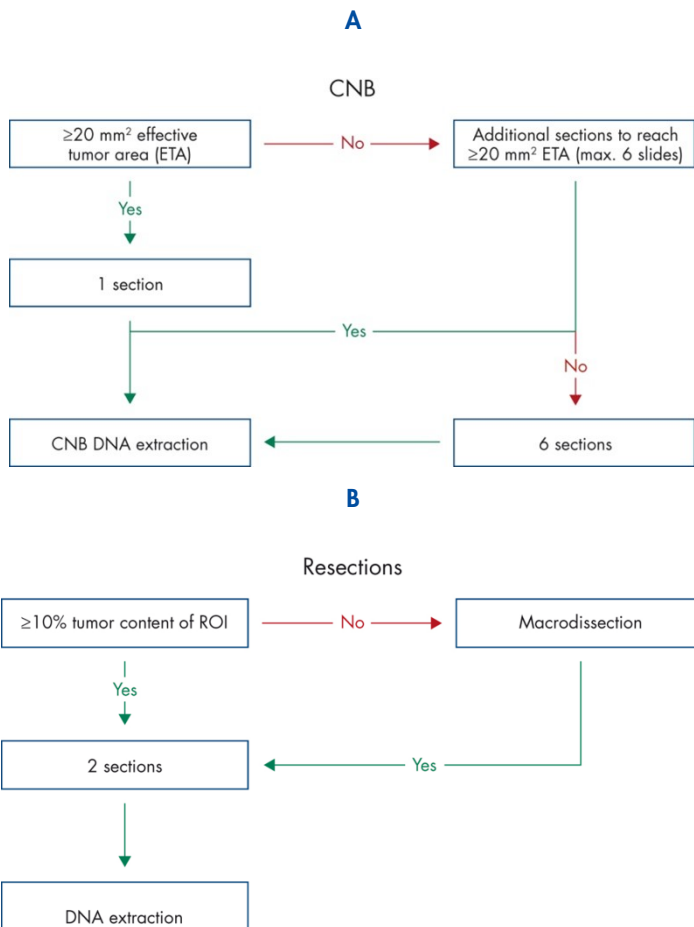


Figure 4. Clinical specimen purification workflow to be used with the *therascreen* PIK3CA RGQ PCR Kit. A: FFPE CNB. B: FFPE tumor tissue resected specimens.

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 4 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)	24 months
Extracted ctDNA	Freezer (–30 to –15°C)	4 weeks

Protocol: DNA extraction

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

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

FFPE tissue resection (RES) specimens

- If RES specimens have $\geq 10\%$ tumor content in the region of interest (ROI), scrape the entire tissue area from 2 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 2 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.

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

- 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 6 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 6 sections, proceed with testing using 6 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 DNA FFPE Tissue Kit. Do not use any other DNA extraction product.

Carry out the DNA extraction according to instructions 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).

Note: The extracted DNA should be within the working Control Ct range (≥ 24.68 and ≤ 31.68) for the sample to be valid.

- 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.

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

Protocol: 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 4 runs using the PIK3CA Reaction Mix available in each kit. The optimal use is 4 runs, with each run containing a maximum of 6 samples. Smaller sample batch sizes will mean that fewer samples can be tested with the *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

1. Ensure that runs are performed using the Rotor-Gene AssayManager version 2.1, Gamma MDx Plug-in, and either the "therascreen_PIK3CA_FFPE_ MDx Assay Profile version 1.0.1 (tissue specimens) or "therascreen_PIK3CA_Plasma_ MDx Assay Profile version 1.0.2 (plasma specimens). Ensure that the relevant software is installed before first use of the Rotor-Gene Q MDx (US) instrument and follow appropriate instructions on run start

and data analysis (see "Performing a PIK3CA mutation analysis run" on page 38).

2. 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 ambient temperature (15–25°C), mixed by inverting 10 times, and centrifuged briefly to collect the contents at the bottom of the tube.
3. Ensure that the PCR loading block is appropriately decontaminated (see "General precautions" on 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 ambient temperature (15°C–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 6 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, 1 PIK3CA Positive Control reaction and 1 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 (µL) of reaction mix (n* + 3)	Volume (µL) of
		Taq DNA polymerase (n* + 3)
Tube RM 1	$19.83 \times (n + 3)$	$0.17 \times (n + 3)$
Tube RM 2	$19.83 \times (n + 3)$	$0.17 \times (n + 3)$
Tube RM 3	$19.83 \times (n + 3)$	$0.17 \times (n + 3)$
Tube RM 4	$19.83 \times (n + 3)$	$0.17 \times (n + 3)$
Tube RM 5	$19.83 \times (n + 3)$	$0.17 \times (n + 3)$
Tube RM 6	$19.83 \times (n + 3)$	$0.17 \times (n + 3)$

*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 overage 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 4 tubes; PCR 4-strip tubes not provided) in the loading block according to the layout in Table 5. Do not cap the strip tubes. Immediately add 20 µL of the appropriate master mix to each PCR strip tube.

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

Note: See Table 5 for the tube layout while setting up the reaction mixes.

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

Controls		Sample number							
Assay	PC	NTC	1	2	3	4	5	6	7
Tube RM 1	1	9	17	25	33	41	49	57	
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 μ L (20 μ L master mix prepared according to Table 4, plus 5 μ L NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position.
E: Empty.

- Immediately add 5 μ L Water for No Template Control to the NTC tubes (tube positions 9–14) and cap the tubes.
- Add 5 μ L of each DNA sample to the sample tubes and cap the tubes immediately after adding each sample to avoid sample-to-sample cross-contamination.
- Add 5 μ L PIK3CA Positive Control to the PC tubes (tube positions 1–6) and cap the tubes.
- 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 (US) 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 (US) instrument is maintained.

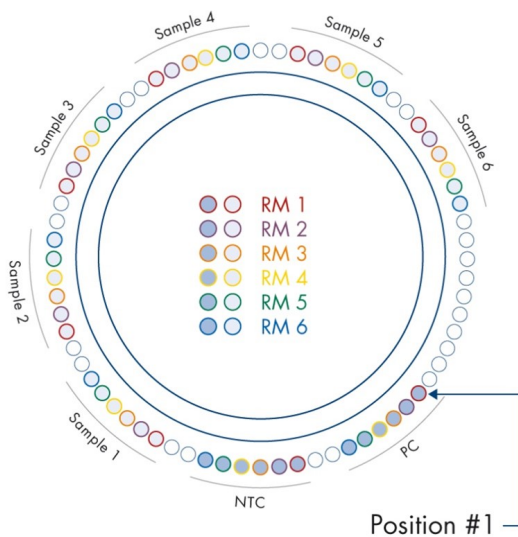
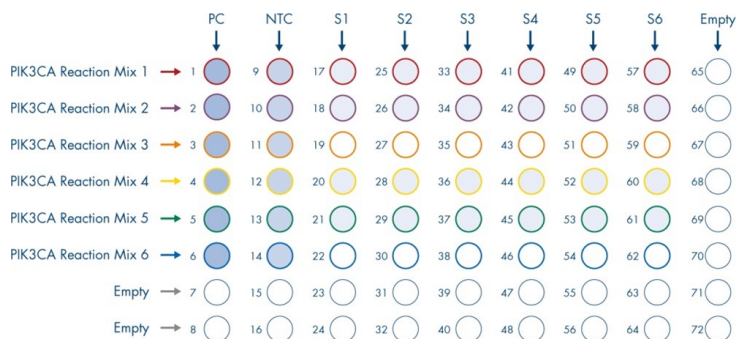


Figure 5. Plate and rotor setup for an experiment with the *therascreen* PIK3CA RGQ PCR Kit. PC: Positive Control. S: DNA sample. NTC: No-template control (water).



Tubes must be inserted into the rotor 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.

Note: Fill all unused positions with capped empty tubes.

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

Performing a PIK3CA mutation analysis run



1. Double-click the Rotor-Gene AssayManager version 2.1 icon on the desktop of the laptop connected to the Rotor-Gene Q MDx (US) instrument.

The Setup environment appears by default.

2. Click **New Manual Worklist** to create a new worklist (Figure 6).

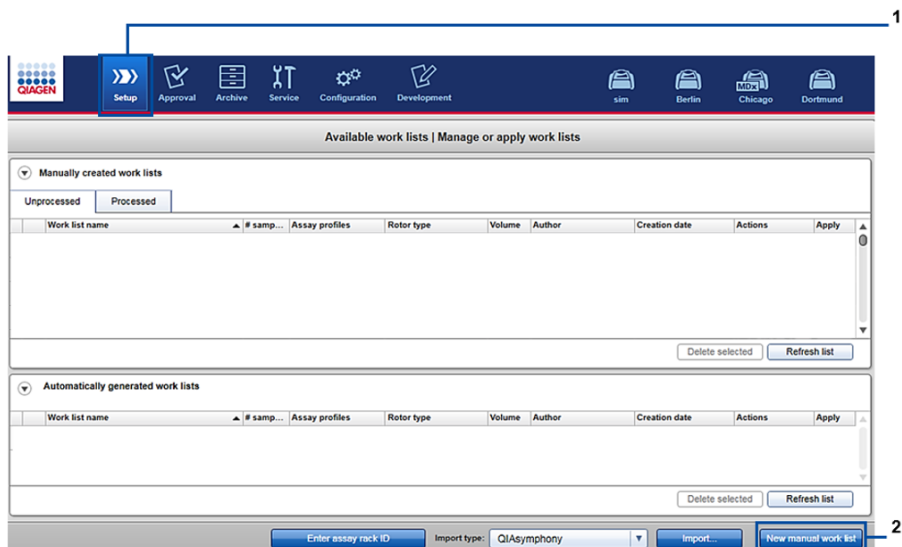


Figure 6. Setting up new manual worklist. 1 = "Setup" tab, 2 = "New manual work list".

3. Select the Assays tab on the left-hand side of the main window. Depending on the sample type, click **therascreen_PIK3CA_FFPE_MDx** Assay Profile for tissue samples or **therascreen_PIK3CA_Plasma_MDx** 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, move the mouse pointer over the assay profile to see the full name (Figure 7).

CAUTION Check that the correct Assay Profile for the specimen type has been selected.



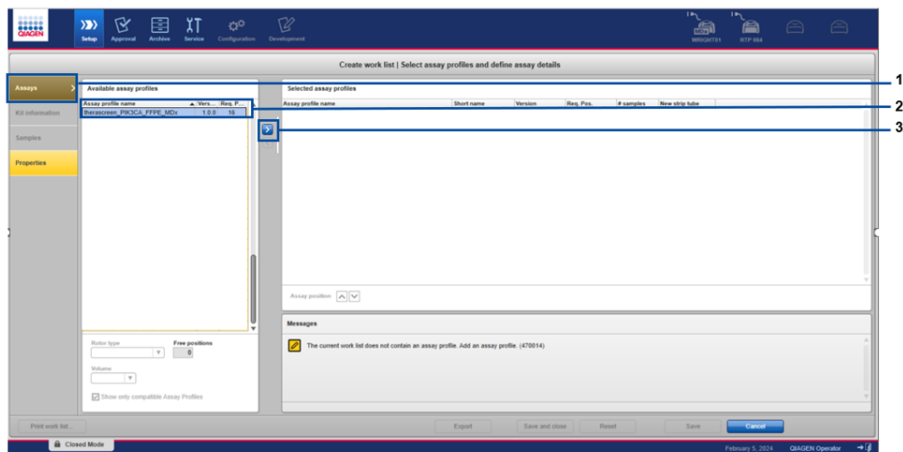


Figure 7. Setting up new manual worklist: Choosing assay profile name. 1 = "Assays" tab, 2 = Available assay profiles with "therascreen_PIK3CA_FFPE_MDx" or "therascreen_PIK3CA_Plasma_MDx" selected, 3 = Select the assay profile.

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

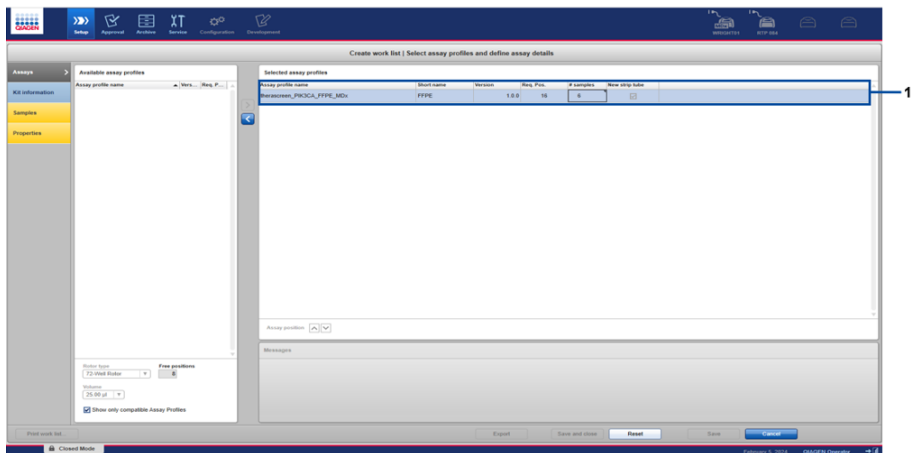


Figure 8. Create work list main window. 1 = Add the number of samples.

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

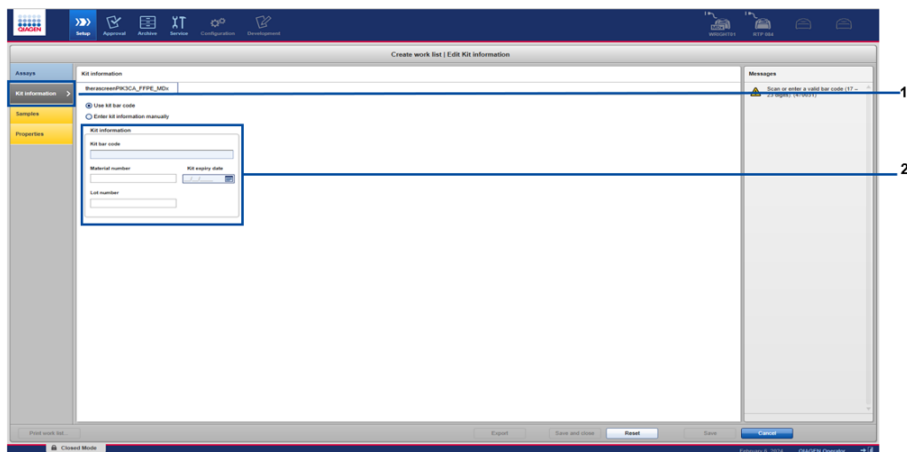


Figure 9. Create work list main window. 1 = “Kit information” tab, 2 = Enter the kit information.

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

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

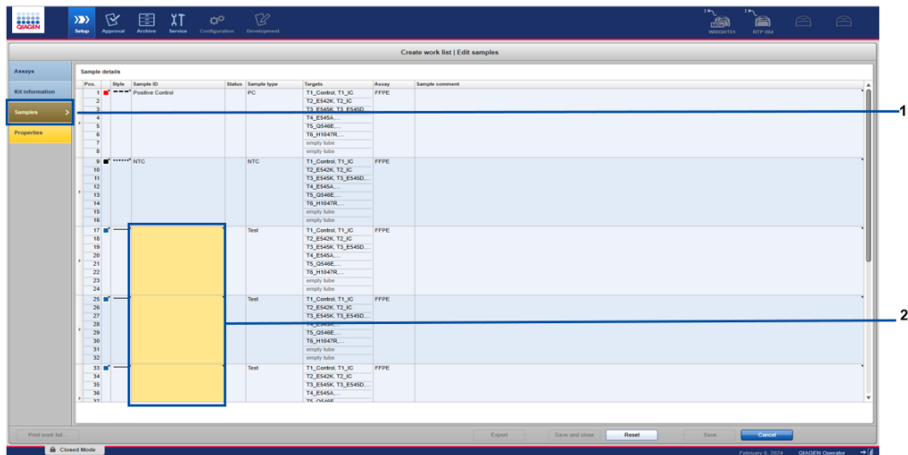


Figure 10. Create work list main window. 1 = "Samples" tab, 2 = Entering sample names.

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

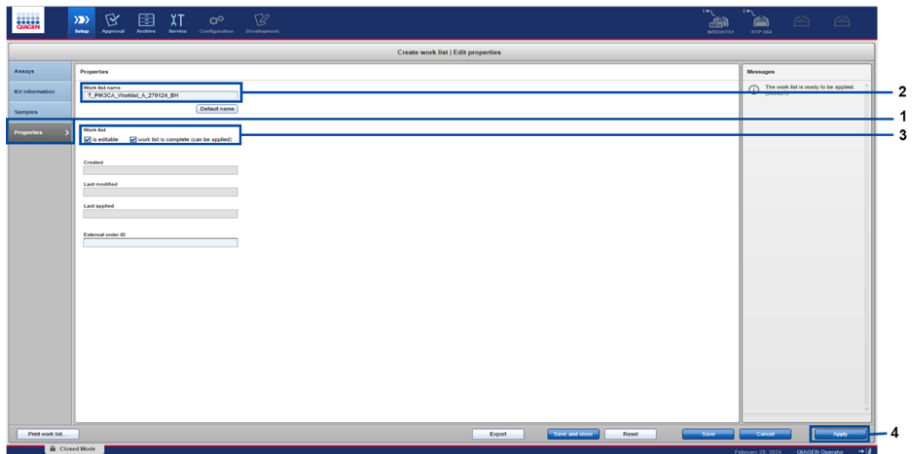


Figure 11. Create work list main window. 1 = "Properties" tab, 2 = Entering worklist name, 3 = Select "is editable" and the "work list is complete", 4 - "Apply".

8. Enter the experiment name into the **Experiment name** field. Select a cyclers from the list of available cyclers and ensure that the **Ring attached** box is checked (Figure 12).

After 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.

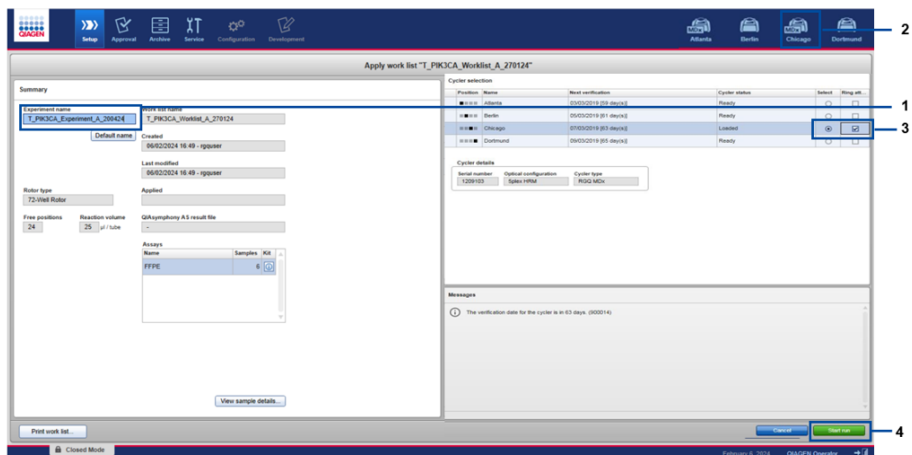


Figure 12. Applying work list and run start. 1 = Enter experiment name, 2 = Instrument selection, 3 = Ensure that "Ring attached" is selected, 4 = Start run.

Note: The Cyclor icon changes its appearance depending on the progress and the result of the run. Full descriptions of these cyclor icons can be found in the *Rotor-Gene AssayManager version 2.1 MDx Core Application User Manual*.

Example cyclor icons are shown in (Figure 13).

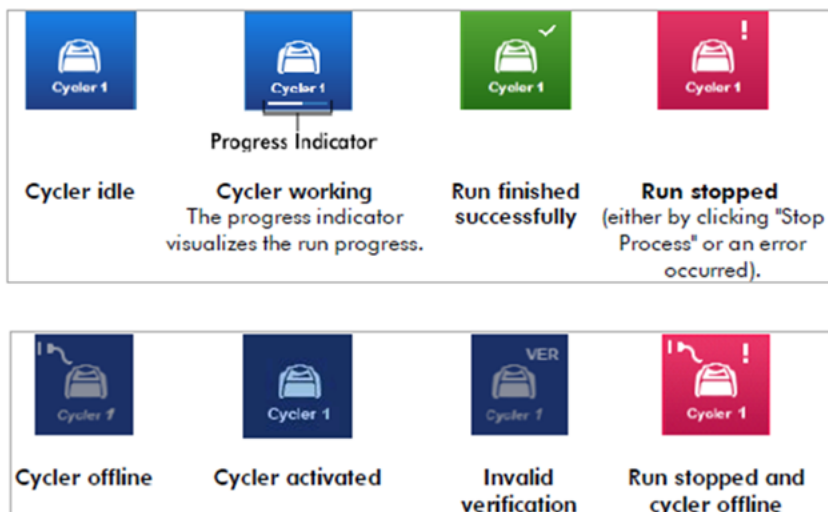


Figure 13. Cycler icons that may be displayed.

9. After the run is complete, click **Finish run**. The "Release and go to approval" dialog box 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 the window when the run is in progress.

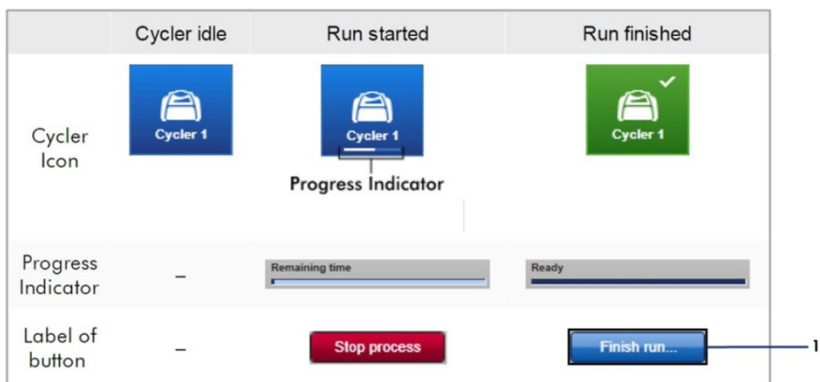


Figure 14. Finishing a run. 1 - "Finish run".

- Click **Release and go to approval** to enter the Approval tab and release the Rotor-Gene Q instrument. 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 (Figure 15).

For a list of potential failures and error codes presented in Rotor-Gene AssayManager, see the *Rotor-Gene AssayManager version 2.1 MDx Core Application User Manual* and *Rotor-Gene AssayManager version 2.1 Gamma MDx (US) Plug-in User Manual*.

✓ **Finish run**

Position: ■ ■ ■ Name: Run status:

Experiment name:

Errors during run:

Comment:

Password:

1

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

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

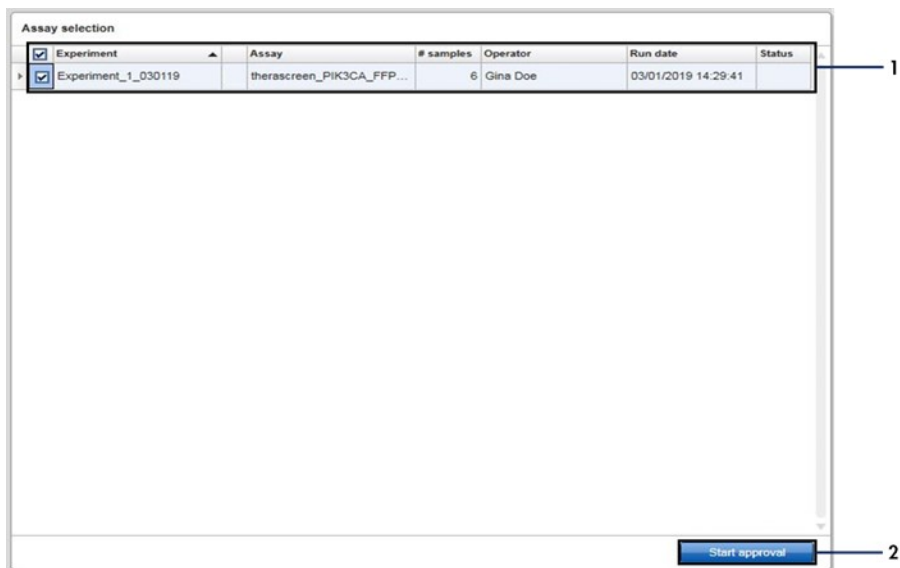


Figure 16. Starting the release process in the "Approval" environment. 1 = Assay selected to approve, 2 = "Start approval".

"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 the "Rotor-Gene AssayManager theascreen PIK3CA Assay Profile flags" on page 54 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.

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

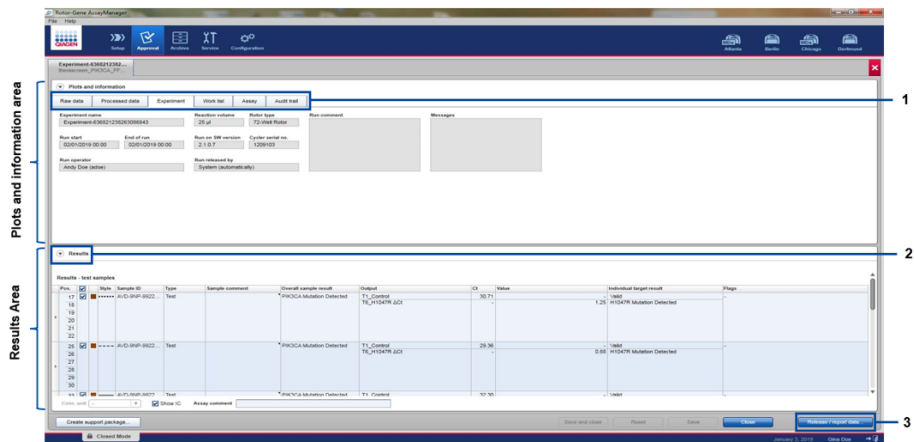


Figure 17. Example of assay results main windows. 1 = “Experiment” tab in the “Plots and information” area. 2 = Results area, 3 = “Release/report data”.

13. 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.

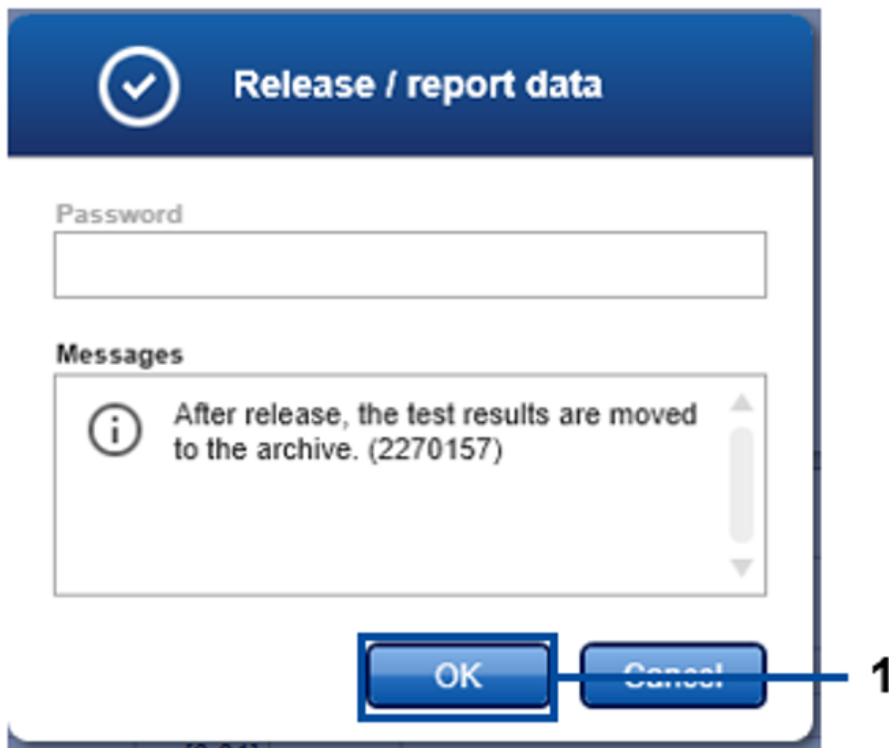


Figure 18. Example of “Release/report data” window.

14. 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 box next to the experiment you would like to view and click **Show assays** (Figure 19).

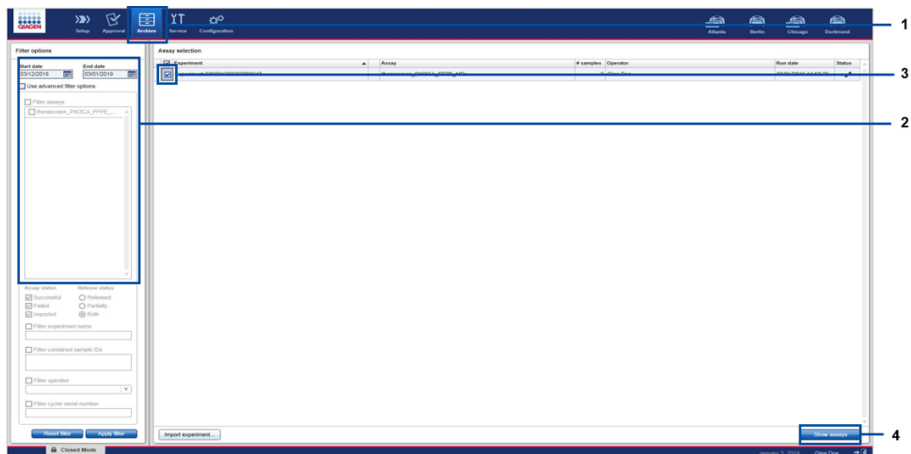


Figure 19. Example of "Experiment Archive" main window. 1 = "Archive" tab, 2 = Search options, 3 = Selecting experiment name, 4 = "Show assays" tab.

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 Ct value. Ct values indicate the quantity of specific input DNA. Low Ct values indicate higher input DNA levels and high Ct values indicate lower input DNA levels. Reactions in which fluorescence crosses the threshold value on or before this Ct value are classed as positive.

By using the Control Reaction to assess the DNA sample it is possible, based on the Ct 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 Ct values allows the *therascreen* PIK3CA Assay Profile to perform a calculation to determine the ΔCt value of the sample using the equation:

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

Based on predetermined analytical Ct and ΔCt 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 Ct values are met and the reactions have been performed successfully.

If the sample Control Ct 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 *therascreen* PIK3CA Assay Profile flags" below.

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 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 software that are defined to set the target result to "INVALID".

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

Rotor-Gene AssayManager *therascreen* PIK3CA Assay Profile flags

All possible flags corresponding to the Rotor-Gene AssayManager version 2.1 Gamma MDx Plug-in are listed in the *Rotor-Gene AssayManager version 2.1 Gamma MDx (US) 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, sample or control affected, and 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. Invalid sample. IC in sample above the specification range.	Repeat run. Retest the sample once; after retest if sample IC Ct 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)_ABOVE_ACCEPTED_RANGE	Invalid run. PC above the specification range.	Repeat run.
(PC)_BELOW_ACCEPTED_RANGE	Invalid run. PC below the specification range.	Repeat run.

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

Flag	Meaning	Action
IC_BELOW_ACCEPTED_RANGE	Invalid run. IC below the specification range in PC or NTC tubes. Invalid sample. IC in sample below the specification range	Repeat run. Retest the sample once; after retest if sample IC Ct 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. Ct value has been detected in NTC.	Repeat run.
NO_CT_VALUE	Invalid PC or IC. No Ct value for PC in PC tubes or for IC in PC and NTC tubes. Invalid sample. No Ct value in sample.	Repeat run. Retest the sample once; after retest if there is still no sample IC Ct, 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.
T1_CONTROL_DNA_INPUT_TOO_HIGH	Invalid sample. Sample Control Ct value below the Control working range.	Sample is too concentrated and must be diluted. Follow instructions in 57.
T1_CONTROL_ABOVE_ACCEPTED_RANGE	Invalid sample. Sample Control Ct value above the Control working range.	Retest the sample once; after retest if the Control Ct value is still above the Control working range, re-extract the sample. If the Control Ct value is still above the Control working range after re-extraction and two rounds of testing, then the sample should be reported as indeterminate

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

Flag	Meaning	Action
T1_CONTROL_NO_CT_VALUE	Invalid sample. No Ct value for sample in sample control tubes.	Retest the sample once; after retest if sample has no Ct, re-extract the sample. If the sample still has no Ct 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 Ct value

There are 3 possible flags for invalid sample due to Control Ct value:

- T1_CONTROL_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 Ct by 1. Samples should be diluted using the water provided in the kit (Water for Dilution [Dil.]).

To calculate the required Control Ct shift (X_R) and estimate the dilution factor required:

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

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

where 25 (for FFPE specimens) or 27 (for plasma specimens) is given the target Control Ct for the diluted sample and X is an actual Control Ct 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

Xr	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

- **T1_CONTROL_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

The specific performance 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 and 1 PIK3CA wild-type (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)

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 3 *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 FFPE LoB results

Exon	Mutation	Base change	LoB (ΔC_T value)	False positive call rate (%)
7	C420R	1258T>C	7.57	1.59
9	E542K	1624G>A	5.09	1.88
	E545A	1634A>C	13.03	0.00
	E545D	1635G>T	9.19	0.53
	E545G	1634A>G	13.03	0.53
	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	1.59
	H1047Y	3139C>T	7.61	0.63

Limit of detection (LoD)

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, 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 3 different *therascreen* PIK3CA RGQ PCR Kit with 24 replicates tested per kit lot per 5–6 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 an LoD Confirmation study (in the case of Q546R) or 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 samples

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	10.00 [†]
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

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 Ct result, which is used to indicate that there is sufficient amplifiable DNA present in the sample. The Control Ct working range was determined using a total of 20 wild-type FFPE clinical

specimens generating 107 data points. The Control Ct working range was set using calculated tolerance intervals. The control reaction Ct range was established as 23.23–33.38 Ct.

ΔCt cutoff values

The assay cutoff value is a specific ΔCt value used to determine whether a sample is classed as positive or negative for a PIK3CA mutation. Samples that generate ΔCt values at or below the cutoff are classified as PIK3CA mutation positive (i.e., PIK3CA Mutation Detected) and ΔCt 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 (ΔCt)
C420R	≤6.0
E542K	≤4.8
E545A	≤10.0
E545D	≤7.5
E545G	≤9.5
E545K	≤6.5
H1047L	≤10.0
H1047R	≤7.0

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

Assay	Cutoff value (Δ Ct)
H1047Y	≤ 6.2
Q546E	≤ 10.0
Q546R	≤ 7.0

Effect of DNA input on Δ Ct values (linearity)

The DNA input level is defined as the total quantity of amplifiable DNA in a sample as determined by the Ct values from the PIK3CA control reaction. To demonstrate that the performance of the *therascreen* PIK3CA RGQ PCR Kit is consistent across the control reaction Ct range (23.23–33.38), a 9-level serial dilution with varying DNA input levels with the upper and lower levels being outside of the Control Reaction Ct working range (23.23–33.38 Ct), 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 Ct 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 Ct range.

The evaluation was performed using one *therascreen* PIK3CA RGQ PCR Kit lot with 3 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 Δ Ct, i.e., there is no statistically significant linear, quadratic, or cubic effect. Overall, the Δ Ct 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 close to 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)

The *therascreen* PIK3CA RGQ PCR Kit is comprised of 6 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 3 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 4 instances of mutant nonspecific amplification between the high MAF sample E545A and H1047L. Overall, 6/240 data points showed mutant nonspecific amplification. The 6 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_MDx” Assay Profile algorithm.

Testing performed post-launch, using synthetic long oligonucleotide and FFPE cell-line specimens, demonstrated cross- reactivity at high MAF. The concentrations and samples tested are not considered representative of those which would be observed in clinical samples. It is possible in rare instances, though extremely unlikely, that multiple mutation calls may be observed when testing clinical samples. In these instances, the mutation call with the lowest Delta Ct value is the correct mutation call.

Interference

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 179 PIK3CA mutant-negative specimens by NGS and 197 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, all except 19 samples had *therascreen* PIK3CA RGQ PCR Kit results that matched the NGS results. The 19 mismatched results were from 15 mutant-negative and 3 mutant-positive samples with less than 5% necrotic content; and 1 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 adipose tissue

Following the same method used to assess the effects of necrosis on the performance of the *therascreen* PIK3CA RGQ PCR Kit, the adipose content was also assessed. FFPE clinical specimens from SOLAR-1 with both *therascreen* PIK3CA RGQ PCR Kit and next generation sequencing (NGS) results were analyzed. A total of 175 PIK3CA mutant-negative specimens by NGS and 197 PIK3CA mutant-positive by NGS were evaluated, which included CNB and RES specimens. Percent adipose, as identified by a pathologist, varied from 0 to 50% for mutant-negative and mutant-positive samples.

For both mutant-positive and mutant-negative FFPE specimens, all except 19 samples had *therascreen* PIK3CA RGQ PCR Kit results that matched the NGS results. Among those, 3 samples had Mutation Detected by NGS but No Mutation Detected by the *therascreen* PIK3CA RGQ PCR Kit. Those samples had % adipose content of less than 10%. Sixteen (16) samples had No Mutation Detected by NGS but were determined Mutation Detected by *therascreen* PIK3CA RGQ PCR Kit. Among those, 9 had % adipose content of less than 10%, 6 had % adipose content of between 10–30%, and 1 had % adipose content between 30–50%. It is therefore unlikely that adipose 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 adipose tissue content up to 50%.

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 Ct 30.00 and then diluted with wild-type (also normalized to Ct 30.00) to give the ΔCt expected at a target MAF level (i.e. 3x LoD or lower). Samples spiked with hemoglobin (endogenous interferent) during the extraction process were not normalized to Ct 30.00 or diluted to the target MAF level 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 Ct 30.00 and dilution to the target MAF level at a concentration representing the highest (worst-case) feasible level of the interfering substance carryover into a sample (10x concentration). In total,

6 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

The *therascreen* PIK3CA RGQ PCR Kit 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 3 lots of the QIAamp DSP DNA FFPE Tissue Kit and 3 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

The reproducibility of the QIAamp DSP DNA FFPE Tissue Kit was examined using sections taken from 11 FFPE specimen blocks; 4 PIK3CA mutant clinical breast cancer specimens, 6 PIK3CA mutant cell-line specimens, and one wild-type clinical breast cancer specimen. For each specimen, extractions were carried out in triplicate by 2 operators, at 3 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

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

To demonstrate repeatability for C420R, E542K, E545A, E545D, E545G, E545K, Q546E, H1047L, H1047R, and H1047Y samples at the 1.5 x LoD mutation level were tested in singlicate with 2 runs per day, by 3 operators across 20 non-consecutive days resulting in 120 data points per target at one site (located in the United Kingdom). To demonstrate repeatability for Q546R samples at 3 mutation levels (LoD, 1.25 x LoD & 1.5 x LoD) were tested in duplicate with 2 runs per day, by 3 operators across 10 days at one site resulting in a total of 120 data points per mutation. For reproducibility, 2 runs per day were performed per operator (3 operators per site) by 2 additional sites (both located in the USA) over 10 days to give an additional 60 data points from each additional site for C420R, E542K, E545A, E545D, E545G, E545K, Q546E, H1047L, H1047R, and H1047Y samples at the 1.5 x LoD level. For Q54R reproducibility, 2 runs per day were performed per operator (3 operators per site) by 2 additional sites (both located in the USA) over 5 days to generate an additional 60 data points for Q546R at the LoD, 1.25 x LoD and 1.5 x LoD levels.

In total for all samples, 240 data points were generated across 3 sites. At each site, samples were tested using 2 *therascreen* PIK3CA RGQ PCR Kit lots (3 lots across 3 sites). Samples were prepared at low DNA input levels where a control Ct value of approximately 30 was targeted.

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	117/120	97.50	92.87
7	C420R	1.5x LoD	120/120	100.00	96.97
9	E542K	1.5x LoD	120/120	100.00	96.97
	E545A	120/120	120/120	100.00	96.97
	E545D	1.5x LoD	120/120	100.00	96.97
	E545G	1.5x LoD	120/120	100.00	96.97
	E545K	1.5x LoD	120/120	100.00	96.97
	Q546E	1.5x LoD	120/120	100.00	96.97
	Q546R	1.5x LoD	119/120	99.17	95.44
20	H1047L	1.5x LoD	119/119	100.00	96.95
	H1047R	1.5x LoD	118/118	100.00	96.92
	H1047Y	1.5x LoD	120/120	100.00	96.97

NA: Not applicable

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	236/239	98.74	96.38
7	C420R	1.5x LoD	238/239	99.58	97.69

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

Exon	Mutation	Mutation level	Fractional proportion of valid results	Correct calls (%)	Lower two-sided 95% CI
9	E542K	1.5x LoD	240/240	100.00	98.47
	E545A	1.5x LoD	240/240	100.00	98.47
	E545D	1.5x LoD	239/239	100.00	98.47
	E545G	1.5x LoD	240/240	100.00	98.47
	E545K	1.5x LoD	239/239	100.00	98.47
	Q546E	1.5x LoD	239/239	100.00	98.47
	Q546R	1.5x LoD	238/239	99.58	97.69
20	H1047L	1.5x LoD	239/239	100.00	98.47
	H1047R	1.5x LoD	237/237	100.00	98.46
	H1047Y	1.5x LoD	239/240	99.58	97.70

NA: Not applicable

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 $\leq 0.78 \Delta Ct$ for 1.5x LoD for all PIK3CA mutations tested in the reproducibility testing. Across all mutant panel members, the SD was $\leq 0.29 \Delta Ct$ for 1.5x LoD for between-lots (lot interchangeability). The SD for within-run variability (repeatability) was $\leq 0.72 \Delta Ct$ for 1.5x LoD.

Cross-contamination/analytical carryover

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 (9 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 mutation assessed across 10 PCR runs; 5 per sample set were set up consecutively by the same operator using the same equipment and Rotor-Gene Q MDx (US) 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 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 373 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	98.5 (194/197)	95.6, 99.7
Negative percent agreement	90.9 (160/176)	85.7, 94.7
Overall percent agreement	94.7 (354/373)	92.2, 96.9

For the 19 overall mutation status discordant results, 3 samples with *therascreen* PIK3CA RGQ PCR Kit negative results had NGS-positive results, while 16 samples with *therascreen* PIK3CA RGQ PCR Kit-positive results gave NGS-negative results. Of the 3 samples with *therascreen* PIK3CA RGQ PCR Kit negative results that had NGS-positive results, 2 were detected by the NGS at MAF levels below the *therascreen* PIK3CA RGQ PCR Kit LoD. Of the 16 samples determined positive by the *therascreen* PIK3CA RGQ PCR Kit and negative by NGS, 9 were low positive (within 1 Δ Ct 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 6 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
H1047L	100.0 (12/12)	73.5, 100.0
H1047R	98.0 (99/101)	93.0, 99.8
Q546E	100.0 (1/1)	2.5, 100.0
Q546R**	0.0 (0/2)	0.0, 84.2

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

**Q546R target detected in another Accuracy study using Sanger Sequencing as orthogonal method.

Clinical performance

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 2 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 170 PIK3CA mutation-positive and 7 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).

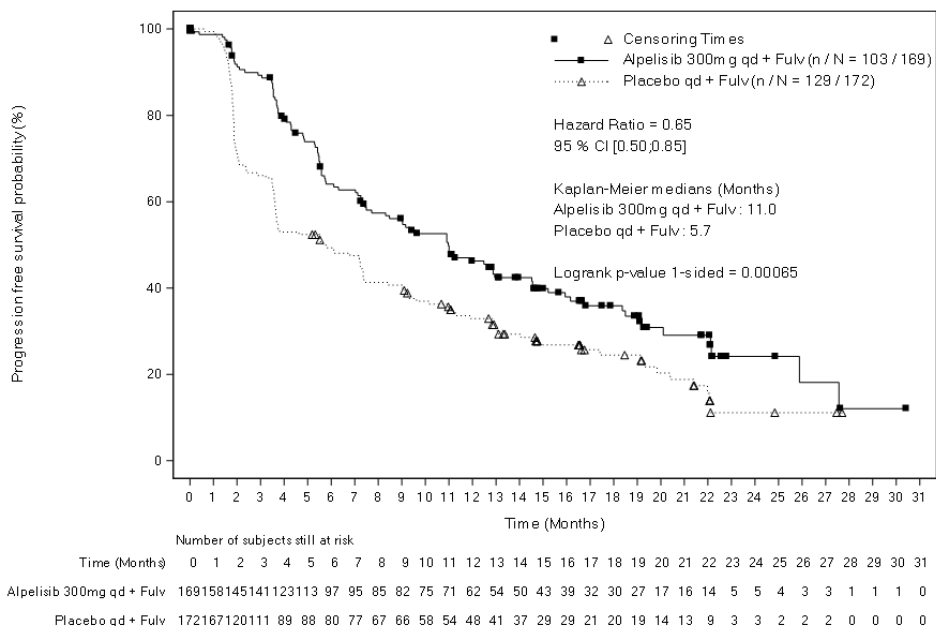


Figure 20. Kaplan-Meier plot of PFS by treatment in the PIK3CA mutant patients randomized to the PIK3CA-mutant cohort of SOLAR-1 by both enrolling assays (CTA or *therascreen* PIK3CA RGQ PCR Kit). Stratified Logrank test and stratified Cox model using strata defined by (i) prior CDK 4/6 inhibitor use and (ii) presence of liver and/or lung metastases. **Note:** From 341 subjects enrolled in the PIK3CA-mutant cohort, 2 were confirmed to be non-mutant following reanalysis with updated software parameters.

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 (344 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. This shows that the clinical efficacy of alpelisib in combination with fulvestrant for the *therascreen* PIK3CA RGQ PCR Kit mutation

positive population was similar to the clinical efficacy in the SOLAR-1 PIK3CA mutant cohort as determined by the enrolling assay (HR for PFS = 0.65; 95% CI: 0.50, 0.85).

Samples from the 395 patients who were randomized using the CTA were retested retrospectively by the *therascreen* PIK3CA RGQ PCR Kit and yielded 385 samples evaluable by *therascreen* PIK3CA RGQ PCR Kit (98.5%) with 6 patient samples not evaluable by *therascreen* PIK3CA RGQ PCR Kit (Table 15) and 4 patient samples excluded from the analysis due to withdrawal of informed consent.

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 = 344 (%)
7	C420R	757	1258 T>C	6 (1.7)
9	E542K	760	1624 G>A	66 (19.2)
	E545A	12458	1634 A>C	4 (1.2)
	E545D	765	1635 G>T	6 (1.7)
	E545G	764	1634 A>G	9 (2.6)
	E545K	763	1633 G>A	91 (26.5)
	Q546E	6147	1636 C>G	1 (0.3)
	Q546R	12459	1637 A>G	0 (0.0)
20	H1047L	776	3140 A>T	24 (7.0)
	H1047R	775	3140 A>G	159 (46.2)
	H1047Y	774	3139 C>T	5 (1.5)

* 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</i> PIK3CA RGQ PCR Kit results	CTA positive (N = 168)	CTA negative (N = 223)	Total (N = 391)
Valid	168 (100.0%)	217 (97.3%)	385 (98.5%)
Positive	163 (97.0%)	11 (4.9%)	174 (44.5%)
Negative	5 (3.0%)	206 (92.4%)	211 (54.0%)
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)	94.9	91.1, 97.4
Overall percent agreement (OPA)	95.8	93.3, 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)	97.6	94.6, 99.2
Overall percent agreement (OPA)	95.8	93.3, 97.6

Table 19 shows the PPA, NPA, and OPA estimates recalculated to adjust for enrichment due to the 9 missing *therascreen* PIK3CA RGQ PCR Kit results in the CTA mutation-negative patients and one missing *therascreen* PIK3CA RGQ PCR Kit result in the CTA mutation-positive 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)	93.5	90.0, 96.9
Negative percent agreement (NPA)	97.7	95.6, 99.5
Overall percent agreement (OPA)	95.8	93.8, 97.7

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 6 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 36% lower risk of disease progression or death (HR = 0.64; 95% CI [0.48, 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 6 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 = 0.70; 95% CI: 0.44, 1.13).

Performance Characteristic: Plasma Specimens

Analytical performance

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)

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, it 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 6 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 plasma LoB results

Exon	Mutation	Base change	LoB (ΔCt)	False positive 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
20	H1047L	3140A>T	15.55	1.89
	H1047R	3140A>G	11.93	0.63
	H1047Y	3139C>T	9.89	0

Limit of detection (LoD)

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 3 different *therascreen* PIK3CA RGQ PCR Kit lots with 24 replicates tested per kit lot per level and in the case of the Q546R mutation was verified

within the Repeatability dataset, across 3 kit lots with a total of 120 replicates. 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	8.44 [†]
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 DNA from cell-line specimens.

[‡] LoD values were verified using clinical plasma specimens.

Genomic DNA input range

The control Ct working range was set using calculated tolerance intervals and LoB values. The control assay Ct 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 Ct working range was set at a Ct value of 24.69 to 31.68 giving a 98% confidence level for 95% of the intended use population.

ΔCt cutoff values

Contrived plasma specimens were used to establish the cutoff values of each mutation. In addition to statistical analysis of ΔCt 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 (ΔCt)
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)

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 (24.69–31.68), 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

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 3 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_MDx Assay Profile algorithm.

Interference

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

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 3 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

To demonstrate that different laboratories will produce acceptable results when starting from the same plasma specimen, extractions were performed across 3 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 3 testing sites (one internal QIAGEN site in the United Kingdom and 2 additional external sites in the USA); 6 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 3 sites, the percentage of correct mutation calls for PIK3CA mutation-positive and wild-type specimens was 100%.

Repeatability and reproducibility

The repeatability of the *therascreen* PIK3CA RGQ PCR Kit was investigated by testing DNA extracted from cell-line specimens, representing all 5 mutations detected by the *therascreen* PIK3CA RGQ PCR Kit at 2x LoD or lower in the case of Q546R.

Repeatability was assessed by testing these samples at 1 site across 10 non-consecutive days for the wild-type samples and 20 non-consecutive days for the mutant samples, using 3 Rotor-

Gene Q instruments and by 3 operators to generate a total of 120 replicates per sample (Table 23).

Table 23. Repeatability overall correct call summary per MAF level

Mutation	Template	Fractional proportion	Percentage (%)	Two-sided lower 95% confidence limit (%)
C420R	2x LOD	120/120	100.00	96.97
E542K	2x LOD	120/120	100.00	96.97
E545K	2x LOD	117/120	97.5	92.87
H1047R	2x LOD	119/119	100.00	96.95
Q546R	LOD	119/120	99.17	95.44
WT	Ci30	116/119	97.48	92.81

Reproducibility was measured by testing contrived samples at 2x LoD level (or lower in the case of Q546R) samples across 3 different sites (one internal QIAGEN site in the United Kingdom and 2 additional external sites in the USA). All of these samples were tested at each site across 5 non-consecutive days for the wild-type samples and 10 non-consecutive days for the mutant samples, using 3 Rotor-Gene Q instruments and by 3 operators to generate a total of 240 replicates per sample (Table 24).

Table 24. Reproducibility overall correct call summary per MAF level

Mutation	Template	Fractional proportion	Percentage (%)	Two-sided lower 95% confidence limit (%)
C420R	2x LOD	237/237	100.00	98.46
E542K	2x LOD	240/240	100.00	98.47
E545K	2x LOD	236/239	98.74	96.38
H1047R	2x LOD	238/238	100.00	98.46
Q546R	LOD	235/238	98.74	96.36
WT	Ci30	232/238	97.48	94.59

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 $\leq 1.17 \Delta Ct$ for 2x LoD for all PIK3CA mutations tested in the reproducibility testing. Across all mutant panel members, the SD was $\leq 0.08 \Delta Ct$ for 2x LoD for between-lots (lot interchangeability). The SD for within-run variability (repeatability/precision) ranged from 0.386–1.163 ΔCt for 2x 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 4 donors (8 tubes per donor). Contrived blood specimens were generated by spiking PIK3CA H1047R mutant fragmented cell-line DNA into blood tubes from 2 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 ΔCt 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 4 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 544 clinical plasma specimens from the SOLAR-1 clinical trial.

DNA samples with both NGS and the *therascreen* PIK3CA RGQ PCR Kit valid results (534/544 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	97.33 (146/150)	93.31
Negative percent agreement	91.15 (350/384)	87.85
Overall percent agreement	92.88 (496/534)	90.36

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.
- Thirty-four 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% (80/82)	91.5, 99.7

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

Clinical performance

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 323 *therascreen* PIK3CA RGQ PCR Kit tissue-positive patients, 177 were *therascreen* PIK3CA RGQ PCR Kit plasma-positive. Of the *therascreen* PIK3CA RGQ PCR Kit tissue-negative patients, 208 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</i> PIK3CA RGQ PCR Kit plasma	<i>therascreen</i> PIK3CA RGQ PCR Kit tissue			
	Positive	Negative	Invalid	Total
Positive	177	6	1	184
Negative	146	208	5	359
Invalid	0	0	0	0
Total	323	214	6	543

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 54.8%, 97.2%, and 71.7%, 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	54.8% (177/323)	(49.2%, 60.3%)
Negative percent agreement	97.2% (208/214)	(94.0%, 99.0%)
Overall percent agreement	71.7% (385/537)	(67.7%, 75.5%)

*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 6 discordant *therascreen* PIK3CA RGQ PCR Kit plasma-positive, *therascreen* PIK3CA RGQ PCR Kit tissue-negative patients, 5 were confirmed plasma-positive by NGS.

Progression-free survival (PFS) analysis

PFS for PIQRAY (alpelisib) in combination with fulvestrant for the PIK3CA *therascreen* PIK3CA RGQ PCR Kit plasma-positive population (N = 183) was observed in the favor of the PIQRAY (alpelisib) plus fulvestrant arm compared to the placebo plus fulvestrant arm with an estimated 45% risk reduction in disease progression or death (HR = 0.55, 95% CI: 0.34, 0.99) (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 0.64 (95% CI: 0.48, 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</i> PIK3CA RGQ PCR Kit plasma-positive (183)	0.55 (0.34, 0.91)

* HR and 95% CI calculated using enrichment adjustment

The PFS HR for the 177 *therascreen* PIK3CA RGQ PCR Kit tissue-positive *therascreen* PIK3CA RGQ PCR Kit plasma-positive patients was 0.5 (95% CI: 0.34, 0.86). Median PFS was 10.9 months for the PIQRAY (alpelisib) plus fulvestrant arm versus 3.7 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 qd + fulv N=89	Placebo qd + fulv N=88	HR (95% CI) PIQRAY 300 mg qd + fulv/placebo qd + fulv
No of events (%)	57 (64.0)	71 (80.7)	0.55 (0.34, 0.86)
PD (%)	55 (61.8)	66 (75.0)	–
Death (%)	2 (2.2)	5 (5.7)	–
No. censored (%)	32 (36.0)	17 (19.3)	–
Median (95% CI)	10.9 (7.0, 16.2)	3.7 (2.0, 6.1)	–

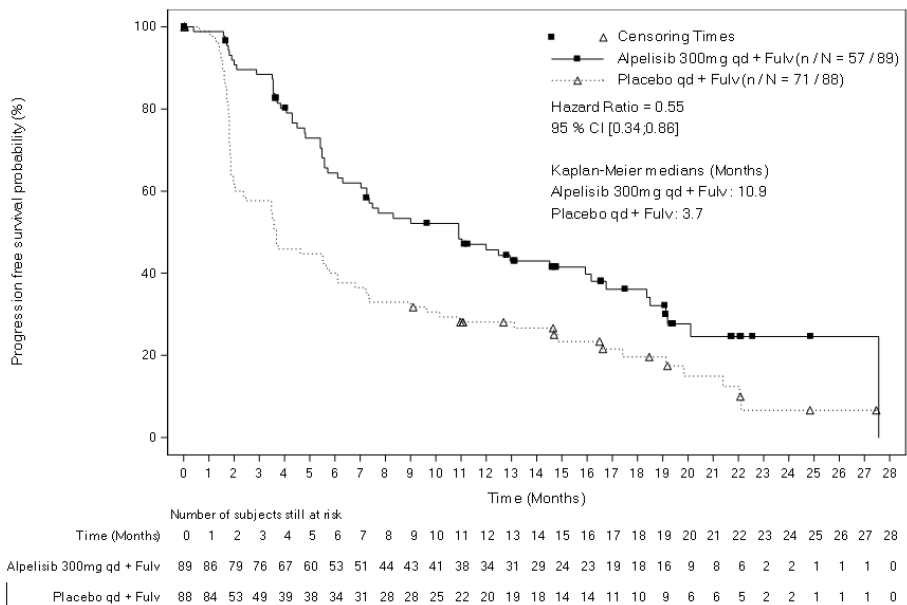


Figure 21. Kaplan-Meier plot of PFS by treatment in *theascreen* PIK3CA RGQ PCR Kit tissue-positive *theascreen* PIK3CA RGQ PCR Kit plasma-positive patients. Hazard ratio obtained with stratified Cox model adjusted for clinically relevant covariates using strata defined by (i) prior CDK 4/6 inhibitor use (ii) presence of liver and/or lung metastases. Stratified Logrank test not performed since the test does not allow for adjusting for the clinically relevant covariates.

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 FFPE plasma specimens N = 184 (%)
7	C420R	757	1258 T>C	2 (1.1)
9	E542K	760	1624 G>A	22 (12.0)
	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 (26.1)
	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	100 (54.3)
	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 97.2% and demonstrated a low risk of false-

positives. A false-negative result may prevent a patient from accessing a potentially beneficial drug. There was 54.8% PPA for plasma/tissue that indicates that plasma-negative patients may be PIK3CA-positive by tissue. Therefore, for confirmation, patients' tissue should be tested where patients' plasma showed PIK3CA negative results with the *therascreen* PIK3CA RGQ PCR Kit.

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 45% risk reduction in disease progression or death compared to placebo plus fulvestrant (HR = 0.55, 95% CI: 0.34, 0.99).

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 (for contact information, visit www.qiagen.com). The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit www.qiagen.com).

Comments and suggestions	
'NO Ct value' flag in Positive Control	
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" on page 22.	Check the storage conditions (see the kit label) of the reagents and use a new kit, if necessary
'Unexpected Ct value' flag in NTC	
a. Contamination occurred during preparation of the PCR	Ensure that 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	
a. Error during preparation of the PCR	Repeat the PCR ensuring accurate pipetting.
'DNA input too high' flag in the sample tube	
a. Sample is too concentrated	Dilute the sample to increase Ct value. Samples should be diluted using the water provided in the kit (Water for Dilution [Dil.]).

'Above acceptable range' flag in sample tube

a. 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 2 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.

Comments and suggestions

'IC above acceptable range' flag in sample tube

a. 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 2 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 Ct value' flag in T1 Control (sample)

a. 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 2 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.

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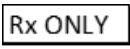
Contact Information

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Symbols

The following symbols appear in the instructions for use or on the packaging and labeling:

Symbol	Symbol definition
	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number (i.e., component labeling)
	Global Trade Item Number
	Unique Device Identifier
	Contains
	Component

Symbol	Symbol definition
	Number
Rn	R is for revision of the Instructions for Use and n is the revision number
	Temperature limitation
	Manufacturer
	Consult instructions for use
	Keep away from sunlight
	Prescription use only
	Warning/Caution

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> 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	873121
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 (US) and accessories		
Rotor-Gene Q MDx System (US)	Real-time PCR cycler with 6 channels, laptop computer, software, accessories, 1- year warranty on parts and labor, installation and training	9002036
PCR Tubes, 0.2 mL (1000)	1000 thin-walled tubes for 1000 reactions of 20–50 µL	981005
Strip Tubes and Caps, 0.1 mL (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Loading Block 72 x 0.1 mL Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 mL tubes	9018901
72-Well Rotor	Loading Block 96 x 0.2 mL PCR Tubes	9018903
Locking Ring 72-Well Rotor	For locking Strip Tubes and Caps, 0.1 mL in the 72-Well Rotor	9018904

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit Instructions for Use. QIAGEN kit Instructions for Use are available at

www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Revision	Description
R1, May 2019	Initial release

Revision	Description
R2, June 2026	Updated to show "<i>therascreen</i>" in italics font throughout the document with the exception of Assay Profile names Updated the PIK3CA RGAM profile version references throughout Updated to remove "or later" throughout the document with reference to RGAM v2.1 software and Assay Profile names Update to Limitations of Procedure section Updated information about breast cancer in the Summary and Explanation of the Test section Update to General Precautions: • Addition of warning for <i>Taq</i> DNA Polymerase (contains Ethoxylated nonylphenol) and associated pictogram, hazards and advice for handling and disposal.) Update to Specimen Handling and Stability: • Revised reference to <i>QIAamp DSP DNA FFPE Tissue Kit Handbook</i> • Updated information in Table 2 • Updated freezer temperature for qDNA extracted from FFPE tissue • Updated information in Table 3: ◦ Revised recommended storage time for plasma at –80°C: 24 months Update to Detection of PIK3CA Mutations protocol: • Minor update to Table 4 format • Replaced RGAM v2.1 screenshots throughout with higher resolution • Minor updates to wording in Procedure section to standardize with QIAGEN vernacular Corrections to Troubleshooting and Interpretation: • Corrected 2 flag names in Table 6 as they appear in RGAM: ◦ T1_Control_DNA_INPUT_TOO_HIGH ◦ T1_Control_ABOVE_ACCEPTED_RANGE • Corrected explanation of Control Ct value: ◦ There are 3 possible flags for invalid sample due to Control Ct value

Revision

Description

Updates to Performance Characteristics for FFPE Tissue section:

- Table 8 Clarification of sample type in title and data updated
- Limit of detection (LoD):
 - Update to mention that false positive rates were verified in an LoD confirmation study for Q546R or the Repeatability and Reproducibility Study
 - Table 9. LoD updated for Q546R
 - Effect of DNA input on ΔCT values (linearity)
 - Clarification that Q546R samples close to LoD may be called false negative at high DNA input
- Cross Reactivity:
 - Added clarification on cross-reactivity handling in high MAF samples:
 - Concentrations and samples not considered representative of clinical samples.
 - In rare cases of multiple mutation calls for clinical samples, the lowest Delta CT value is the correct mutation call.
- Interference:
 - Updated effects of adipose tissue
 - Effects of hemoglobin and exogenous substances:
 - Clarification that samples were diluted to the target Mutant Allele Frequency (MAF)
- Repeatability and reproducibility:
 - Updated the mutation levels tested for the PIK3CA mutants in relation to the respective LoDs: one level for C420R, E542K, E545A, E545D, E545G, E545K, Q546E, H1047L, H1047R and H1047Y), three levels for Q546R,
 - Updated the number of runs performed, sites and operators employed and study duration.
 - Data updated for:

Revision

Description

- Table 11: Repeatability for Wild Type and Q546R (table title updated also)
- Table 12: Reproducibility for Wild Type, Q546E and Q546R
- Accuracy: Comparison to the analytical reference method:
- Clarification of sample type in section title
- Data and/or information details updated for Table 13 and Table 14
- Clinical performance:
 - Added details of randomization of patients in the SOLAR-1 study
 - Caption for Figure 20 updated to clarify enrolment of patients and note added to show the change of status for two patients following the re-analysis with updated software parameters.
 - Added clarification regarding clinical data outcome following reanalysis with updated software parameters
 - Summary of PFS survival paragraph moved from Page 81 to Page 79 and updated following reanalysis with updated software parameters
 - Titles of Table 17, Table 18 and Table 19 updated for clarity of the description

Updates to Performance Characteristics for Plasma section:

- Clarification of Plasma Specimens as sample type in main section title
- Limit of blank (LoB):
 - Data updated in Table 20 and corrected typographical error in title
 - Limit of detection (LoD):
 - Data updated for Q546R in Table 21
- Repeatability and reproducibility:
 - Typographical error for mutation C420R corrected in Table 23 and Table 24,
- Accuracy: Comparison to the analytical reference method (Plasma specimens):
 - Number of specimens and valid data updated according to the SOLAR-

Revision

Description

1 clinical trial

- Clinical performance:
 - Data in Figure 21 updated to align with those shown in Table 30
 - Data updated in Table 31
 - Added clarification regarding clinical data outcome following reanalysis with updated software parameters
 - Updated summary of PFS survival following reanalysis with updated software parameters

Revised References section

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