January 2020

PIK3CA RGQ PCR Kit
Instructions for Use (Handbook)

24

Version 1

For research use only

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

873101


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Sample to Insight

QIAGEN

Contents

Intended Use

The PIK3CA RGQ PCR Kit is a real-time PCR test for the qualitative 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) tumor tissue or circulating tumor DNA (ctDNA) isolated from plasma derived from K_2EDTA anticoagulated peripheral venous whole blood.

FFPE tumor specimens are recommended to be processed using the QIAamp® DSP DNA FFPE Tissue Kit or the QIAamp DNA FFPE Tissue Kit for manual sample preparation. K_2EDTA anticoagulated whole peripheral venous blood plasma specimens are recommended to be processed using the QIAamp DSP Circulating Nucleic Acid Kit or QIAamp Circulating Nucleic Acid Kit for manual sample preparation. For both specimen types, the Rotor-Gene Q 5plex HRM instrument is used for automated amplification and detection.

The test is for research use only.

Limitations of the Procedure

This Instructions for Use document must be completely read and understood before the PIK3CA RGQ PCR Kit is used.

Samples with no detected amplification may harbor *PIK3CA* mutations not detected by the PIK3CA RGQ PCR Kit.

Analytical performance of the Kit when detecting the following *PIK3CA* mutations: E545A, E545D, Q546E, Q546R, and H1047Y, was established using contrived plasma specimens (cell-line DNA spiked into plasma) only, not using clinical specimens.

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 insufficient or the concentration is too high for mutation analysis.

The 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 can be detected by the PIK3CA RGQ PCR Kit, no amplification will be detected.

It is not known whether the PIK3CA RGQ PCR Kit shows cross-reactivity (leading to a result of "Amplification Detected") to additional *PIK3CA* mutations besides those listed as biomarkers detected by the kit.

The 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 PIK3CA RGQ PCR Kit should be used with all reaction mixes.

The impact of microbial contamination on the performance of the PIK3CA RGQ PCR Kit is unknown. Operators must exercise due caution to avoid introduction of microbial contaminants during testing procedures and should not use the kit components if evidence of microbial growth is observed.

The PIK3CA RGQ PCR Kit is recommended for use with DNA extracted from FFPE tumor tissue or from plasma specimens prepared from K_2EDTA anticoagulated whole peripheral venous blood.

The PIK3CA RGQ PCR Kit is recommended for use with the QIAamp DSP DNA FFPE Tissue Kit or the QIAamp DNA FFPE Tissue Kit (for tumor tissue specimens), or the QIAamp DSP Circulating Nucleic Acid Kit or the QIAamp Circulating Nucleic Acid Kit (for plasma specimens).

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

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

Strict compliance with the *PIK3CA RGQ PCR Kit Instructions for Use (Handbook)* is required for optimal results. Dilution of the reagents is not recommended and will result in a loss of performance.

The instructions provided in this handbook are to be used with Rotor-Gene AssayManager software version 2.1, together with the Gamma Plug-in version 1.0.0 and PIK3CA_Tissue_RUO Assay Profile version 1.0.1. or PIK3CA_Plasma_RUO Assay Profile version 1.0.0.

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 PI3Kα 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).

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

The test is for research use only.

Table 1. 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 PIK3CA RGQ PCR Kit is comprised of six separate PCR amplification reaction mixes:

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

The principal components of the kit are explained below:

Mutation reaction mixes

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

ARMS

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

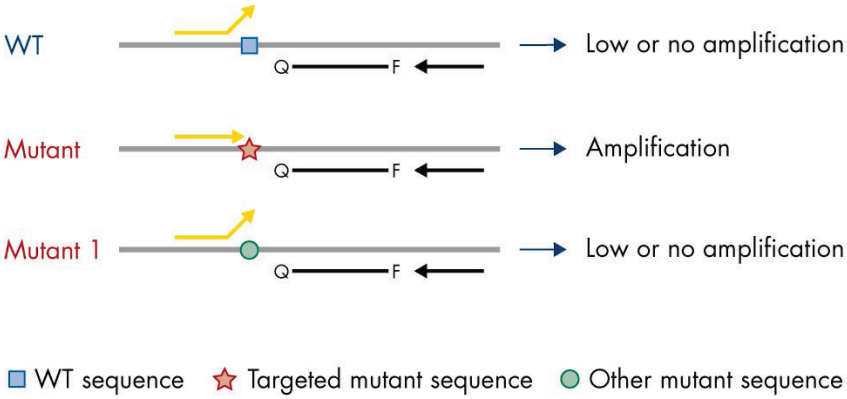


Figure 1. Identification of specific mutation by ARMS PCR. WT: Wild type. Q—F: Double-dye probe. ⇆: Forward and reverse primers.

Hydrolysis probes

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

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

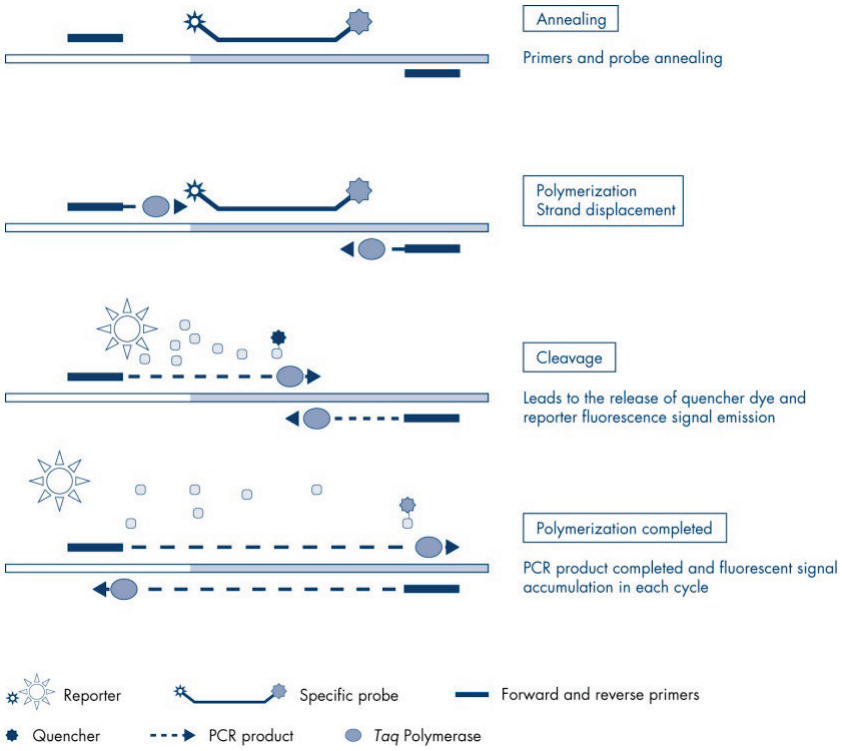


Figure 2. Reaction principle with hydrolysis probes.

PCR clamp

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

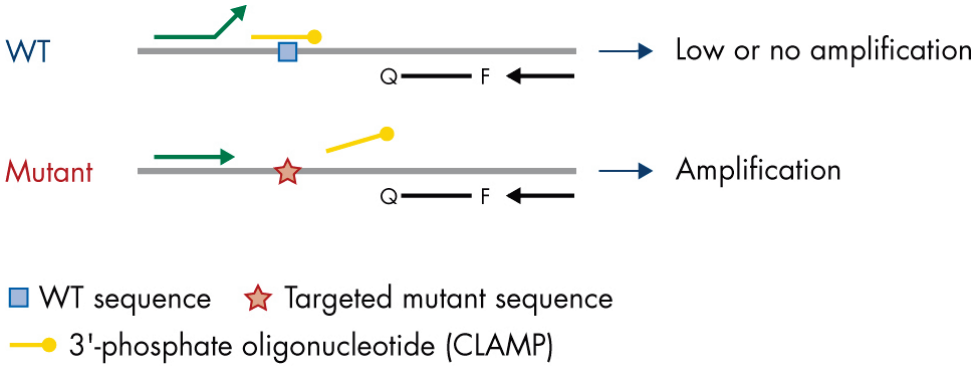


Figure 3. PCR clamp technology. WT: Wild type. Q—F: Double-dye probe. ⇆: Forward and reverse primers.

Control reaction

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

Internal control

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

Positive control

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

Negative control

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

Sample diluent

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

Platform and Software

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

- Rotor-Gene AssayManager® version 2.1
- Gamma Plug-in version 1.0.0
- PIK3CA_Tissue_RUO Assay Profile version 1.0.1 for analysis of gDNA from FFPE tumor tissue specimens
- PIK3CA_Plasma_RUO Assay Profile version 1.0.0 for analysis of ctDNA from plasma specimens

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

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

Run parameters

The Rotor-Gene Q 5plex HRM instrument is programmed for different cycle parameters (or "runs") with the 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

PIK3CA RGQ PCR Kit (24)		
Catalog no.		**873101**
Number of reactions		**24**
Contents	**Cap color**	**Volume**
PIK3CA Reaction Mix 1	Red	750 µl
PIK3CA Reaction Mix 2	Purple	750 µl
PIK3CA Reaction Mix 3	Orange	750 µl
PIK3CA Reaction Mix 4	Yellow	750 µl
PIK3CA Reaction Mix 5	Green	750 µl
PIK3CA Reaction Mix 6	Blue	750 µl
Taq DNA Polymerase (*Taq*)	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 Recommended but Not Provided

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

Reagents

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) or QIAamp DNA FFPE Tissue Kit (QIAGEN, cat. no. 56404); see "DNA extraction from FFPE tumor tissue specimens",

  or

  QIAamp DSP Circulating Nucleic Acid Kit (QIAGEN, cat. no. 61504) or QIAamp Circulating Nucleic Acid Kit (QIAGEN, cat. no. 55114); see "DNA extraction from plasma specimens".
- DNA*Zap*™ PCR-degrading solutions
- Distel High Level Laboratory Disinfectant and isopropyl alcohol (IPA) wash

Consumables

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

Equipment

- Permanent marker
- Rotor-Gene Q 5plex HRM Platform (QIAGEN, cat. no. 9001580) or Rotor-Gene Q 5plex HRM System (QIAGEN, cat. no. 9001650)*
- Rotor-Gene AssayManager v2.1, Gamma Plug-in and "PIK3CA_Tissue_RUO" and/or "PIK3CA_Plasma_RUO" Assay Profile
- Dedicated pipettes* (adjustable) for sample preparation
- Dedicated pipettes* (adjustable) for PCR master mix preparation
- Dedicated pipettes* (adjustable) for dispensing of template DNA
- Benchtop centrifuge* with rotor for 1.5 ml tubes
- Thermomixer*, heated orbital incubator*, heating block*, or water bath* capable of incubation at 56°C, 70°C, and 90°C
- QIAvac 24 Plus vacuum manifold (QIAGEN, cat. no. 19413)
- QIAvac Connecting System (QIAGEN, cat. no. 19419)
- Vacuum Pump (QIAGEN, cat. no. 84010) or equivalent pump capable of producing a vacuum of –800 to –900 mbar
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction set up (QIAGEN, cat. no. 9018901)
- Loading Block 96 x 0.2 ml PCR Tubes, aluminum block for manual reaction setup with a single-channel pipet in 96 x 0.2 ml PCR tubes (QIAGEN, cat. no. 9018905)
- 72-Well Rotor, for holding Strip Tubes and Caps, 0.1 ml, with reaction volumes of 10–50 µl; requires Locking Ring 72-Well Rotor (QIAGEN, cat. no. 9018903)
- Locking Ring 72-Well Rotor, for locking Strip Tubes and Caps, 0.1 ml in the 72-Well Rotor (QIAGEN, cat. no. 9018904)

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

Warnings and Precautions

For research use only.

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

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

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

Tissue specimens only: Recommended for use with the QIAamp DSP DNA FFPE Tissue Kit or QIAamp DNA FFPE Tissue Kit.

For safety information for the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) and the QIAamp DNA FFPE Tissue Kit (cat. no. 56404), see the respective kit handbooks.

Plasma specimens only: Recommended for use with the QIAamp DSP Circulating Nucleic Acid Kit or the QIAamp Circulating Nucleic Acid Kit.

For safety information for the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) and the QIAamp Circulating Nucleic Acid Kit (cat. no. 55114), see the respective kit handbooks.

General precautions

- The test is recommended for use with FFPE tumor tissue specimens or K_2EDTA plasma specimens.
- All chemicals and biological materials are potentially hazardous. FFPE specimen material and nucleic acids prepared from it are unlikely to pose an infection hazard, but all plasma specimens should be treated as potentially hazardous. Local institutional Health and Safety procedures must always be adhered to.
- Discard specimen, sample, and assay waste according to your local safety procedures.
- Reagents for the PIK3CA RGQ PCR Kit are diluted optimally. Dilution of reagents further may result in a loss of performance. Use of reaction volumes (reaction mix plus sample) of less than 25 µl is not recommended.
- All reagents supplied in the PIK3CA RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same PIK3CA RGQ PCR Kit. Substitution of the reagents in the PIK3CA RGQ PCR Kit or between PIK3CA RGQ PCR Kits is not recommended, as this may affect performance.
- We recommend that only the *Taq* DNA Polymerase (Tube *Taq*) that is provided in the PIK3CA RGQ PCR Kit is used. Do not substitute with *Taq* DNA Polymerase from other QIAGEN kits or with *Taq* DNA Polymerase from another supplier, as this may affect performance.
- Refer to the Rotor-Gene Q 5plex HRM instrument user manual for additional warnings, precautions, and procedures.
- Do not use expired or incorrectly stored components.
- Use extreme caution to prevent contamination of the control and reaction mix reagents with the synthetic materials that are contained in the positive control reagent.
- Use extreme caution to prevent cross-contamination between samples. Cap the tubes promptly after addition of each sample.

- Thoroughly decontaminate the loading block before using it for preparation of assay master mixes. The use of DNA*Zap* PCR-degrading solutions followed by Distel High Level Laboratory Disinfectant and IPA wash is recommended. The loading block must be dry before use.
- Use of individual, dedicated pipettes for setting up reaction mixes and adding positive control reagents is recommended.
- We recommend that users 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 5plex HRM instrument until the run has finished.
- Caution must be exercised to ensure correct sample testing to avoid incorrect sample entry, loading errors, and pipetting errors.
- We recommend that the samples are handled in a systematic way and clearly labeled to ensure correct identification at all times.

We recommend the following precautions are taken:

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

Reagent Storage and Handling

Shipping conditions

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

Storage conditions

The 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 PIK3CA RGQ PCR Kit is stable until the stated expiration date.

Stability

Once opened, reagents can be stored in their original packaging at –30 to –15°C for 12 months or until the stated expiry date shown on the packaging. Repeated thawing and freezing should be avoided. We recommend that the PIK3CA RGQ PCR Kit reagents are not subjected to more than five freeze–thaw cycles.

We recommend that the PIK3CA RGQ PCR Kit reagents are thawed at room temperature for a minimum of 1 hour and a maximum of 4.5 hours before use. Once the reagents are ready to use, the PCR reactions can be set up. We recommend that the total setup time from the start of PCR setup to the start of the run should not exceed **7.5 hours** if carried out at ambient temperature.

We also recommend that the Rotor-Gene Q tubes, containing the master mixes and the sample DNA, are loaded onto the Rotor-Gene Q immediately.

Note: The recommended total setup time includes both the PCR setup and storage of prepared reaction mixes.

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 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 Handling and Storage

Specimen handling: FFPE tumor tissue

The PIK3CA RGQ PCR Kit is recommended for use with gDNA extracted from FFPE tumor tissue specimens and core needle biopsy (CNB) specimens.

To prepare tumor tissue specimens for DNA extraction, the following steps are recommended:

- Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides.
- A trained individual should assess a Hematoxylin & Eosin (H&E) stained section for region of interest (ROI) content and effective tissue area (ETA). The stained slide should be marked to determine the ROI. Serial sections should be used 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.

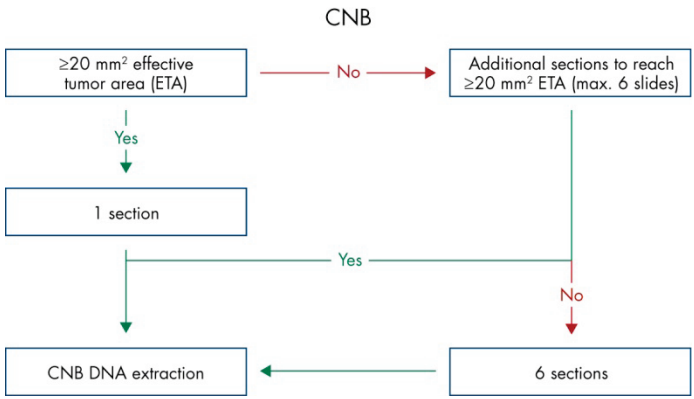
CAUTION 	Use dry scalpels. Do not perform this step in a laminar flow or fume hood.

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

We recommend that tumor specimens, blocks, slides, samples, and microcentrifuge tubes are labeled, handled, and stored ready for extraction in a controlled fashion according to local procedures.

There are two separate workflows that can be used; one when using FFPE tumor tissue specimens and one when using FFPE CNB specimens (Figure 4).

A



B

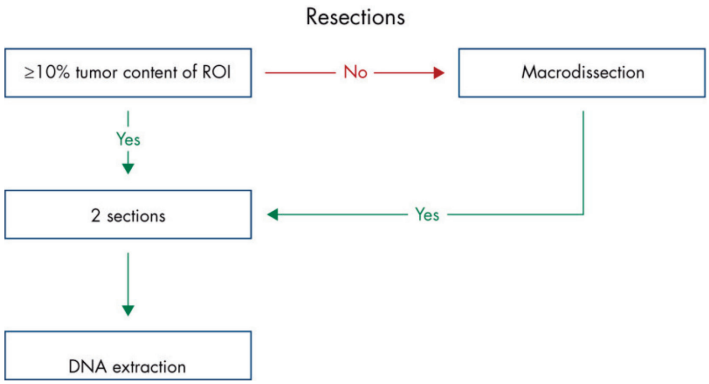


Figure 4. Clinical specimen purification workflow to be used with the PIK3CA RGQ PCR Kit. A: FFPE CNB. **B**: FFPE tumor tissue resection specimens.

Specimen handling: Plasma

The PIK3CA RGQ PCR Kit is recommended for use with DNA isolated from K_2EDTA anticoagulated plasma specimens. All plasma specimens should be treated as potentially infectious.

We recommend that whole peripheral venous blood collected in K_2EDTA blood collection tubes be processed to obtain plasma within four hours of blood collection. Failure to do so may result in genomic DNA contamination of the sample. For further information on the isolation of plasma from whole blood, refer to Appendix A of the *QIAamp DSP Circulating Nucleic Acid Kit Handbook* or the *QIAamp Circulating Nucleic Acid Kit Handbook,* as appropriate.

Plasma specimens should be stored at –80°C. Frozen plasma specimens should be equilibrated to room temperature before use.

We recommend that specimens, samples, and microcentrifuge tubes are labeled, handled, and stored ready for extraction in a controlled fashion, according to local procedures.

Specimen storage

Prior to DNA extraction, we recommend that FFPE blocks and slides be stored at room temperature (15 to 25°C) and plasma be stored at –90 to –65°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 tumor tissue

Storage	Maximum recommended storage time
Freezer (–22 to –18°C)	5 weeks
Refrigerator (2 to 8°C)	1 week
Freezer (–80°C)	33 months

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

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

Procedure

DNA extraction from FFPE tumor tissue specimens

We recommend that gDNA is extracted using the QIAamp DSP DNA FFPE Tissue Kit (cat. no. 60404) or the QIAamp DNA FFPE Tissue Kit (cat. no. 56404).

We also recommend that DNA extraction is carried out according to instructions in the *QIAamp DSP DNA FFPE Tissue Kit Handbook* or the *QIAamp DNA FFPE Tissue Kit Handbook* noting the following:

- Use the number of slides and elution volumes as recommended in the sections below ("FFPE tumor tissue specimens" and "FFPE CNB specimens").
- If the tissue is not pelleted after the first centrifugation, perform an additional centrifugation.
- Use of molecular biology grade ethanol* is recommended 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 tumor tissue specimens

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

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

FFPE CNB specimens

- For CNB specimens, use up to a maximum of six 4–5 µm sections to obtain the minimum required effective tissue area (ETA) of 20 mm^2. Use the minimum number of sections possible to achieve 20 mm^2 ETA.
- For specimens where 20 mm^2 ETA cannot be met with the maximum of six sections, proceed with testing using six sections.
- We recommend that Proteinase K digestion 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 and QIAamp DNA FFPE Tissue Kit), after 10 minutes incubation on the column.

DNA extraction from plasma specimens

We recommend that DNA is extracted using the QIAamp DSP Circulating Nucleic Acid Kit (cat. no. 61504) or the QIAamp Circulating Nucleic Acid Kit (cat. no. 55114) with the stipulations described below for purifying ctDNA from plasma specimens.

Carry out the DNA extraction according to instructions in the *QIAamp DSP Circulating Nucleic Acid Kit Handbook* or the *QIAamp Circulating Nucleic Acid Kit Handbook*.

If the QIAamp DSP Circulating Nucleic Acid Kit is being used, follow the instructions for the 'Classic Protocol' in the *QIAamp DSP Circulating Nucleic Acid Kit Handbook*.

Important points before starting

- The starting volume of plasma is 2 ml.
- Where 2 ml is not available, we recommend that the specimen volume be adjusted to 2 ml using phosphate buffered saline (PBS).

  Note: We recommend that the extracted DNA be within the working Control C_T range ($\geq$24.68 and $\leq$31.68) for analysis to be successful.
- Carry out all centrifugation steps 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 and the QIAamp Circulating Nucleic Acid Kit).
- We recommend that molecular biology grade ethanol* is used for all required steps.
- We recommend that purified ctDNA is stored at –30 to –15°C.
  Note: All assays in the PIK3CA RGQ PCR Kit generate short PCR products. However, the 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.

Detecting *PIK3CA* mutations

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

Important points before starting

- Up to 24 samples can be assessed over four runs using the PIK3CA Reaction Mix available in each kit. The optimal use is four runs, with each run containing a maximum of six samples. Smaller sample batch sizes will mean that fewer samples can be tested with the PIK3CA RGQ PCR Kit.
- The sample should be tested using all reaction mixes provided in the PIK3CA RGQ PCR Kit.
- Analysis of mixed batches of samples derived from both tissue and plasma specimens in the same PCR run is not recommended, as separate PC and NTC controls are required for each specimen type, reducing the number of test samples that can be analysed.
- Do not vortex the *Taq* DNA Polymerase (Tube *Taq*) or any mix containing *Taq* DNA Polymerase, as this may inactivate the enzyme.
- Pipet *Taq* DNA Polymerase by carefully placing the pipette tip just under the liquid surface to avoid the exterior of the tip being coated in excess enzyme.

Things to do before starting

- Ensure that runs are performed using the Rotor-Gene AssayManager v2.1, Gamma Plug-in, and the "PIK3CA_Tissue_RUO" Assay Profile (tumor tissue specimens) or "PIK3CA_Plasma_RUO" Assay Profile (plasma specimens). Ensure that the relevant software is installed before first use of the Rotor-Gene Q 5plex HRM instrument and follow appropriate instructions on run start and data analysis ("Performing a PIK3CA mutation analysis run").

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

Procedure

1. Thaw all reaction mixes, Water for No Template Control, *Taq* DNA Polymerase, PIK3CA Positive Control, and DNA samples at room temperature (15–25°C) for a minimum of 1 hour and up to a maximum of 4.5 hours.
2. After 1 hour, mix all reagents thoroughly by inverting each tube 10 times to avoid localized concentrations of salts. Centrifuge all reagents briefly to collect the contents at the bottom of the tube.

   Note: Do not vortex the *Taq* DNA Polymerase (Tube *Taq*) or any mix containing *Taq* DNA Polymerase, as this may inactivate the enzyme.
3. Label six microcentrifuge tubes (not provided) according to Table 4. Prepare sufficient master mixes (control and mutation reaction mixes) plus *Taq* DNA Polymerase for the DNA samples, one PIK3CA Positive Control reaction and one No Template Control reaction, according to the volumes in Table 4.

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

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

Table 4. Preparation of assay master mixes

Reaction mix tube	Volume of reaction mix (n* + 3)	Volume of *Taq* DNA polymerase (n* + 3)
Tube RM 1	19.83 µl x (n + 3)	0.17 µl x (n + 3)
Tube RM 2	19.83 µl x (n + 3)	0.17 µl x (n + 3)
Tube RM 3	19.83 µl x (n + 3)	0.17 µl x (n + 3)
Tube RM 4	19.83 µl x (n + 3)	0.17 µl x (n + 3)
Tube RM 5	19.83 µl x (n + 3)	0.17 µl x (n + 3)
Tube RM 6	19.83 µl x (n + 3)	0.17 µl x (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 four tubes) 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.

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	E
Tube RM 2	2	10	18	26	34	42	50	58	E
Tube RM 3	3	11	19	27	35	43	51	59	E
Tube RM 4	4	12	20	28	36	44	52	60	E
Tube RM 5	5	13	21	29	37	45	53	61	E
Tube RM 6	6	14	22	30	38	46	54	62	E
E	E	E	E	E	E	E	E	E	E
E	E	E	E	E	E	E	E	E	E

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

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

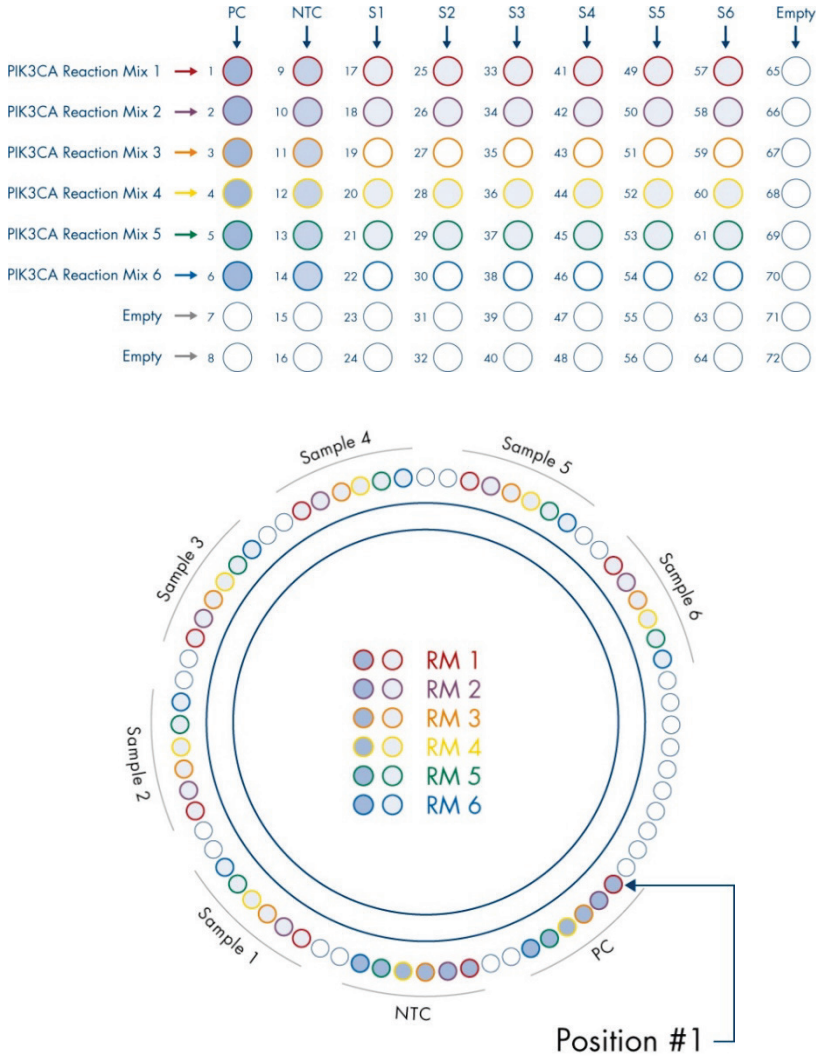


Figure 5. **Plate and rotor setup for an experiment with the PIK3CA RGQ PCR Kit.**
PC: Positive Control. **S**: DNA sample. **NTC**: No-template control (water).

CAUTION 	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.

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

Performing a *PIK3CA* mutation analysis run

13. Double-click the Rotor-Gene AssayManager v2.1 icon on the desktop of the laptop connected to the Rotor-Gene Q 5plex HRM instrument.

14. The Setup environment appears by default. Click **New manual worklist** to create a new worklist (Figure 6).

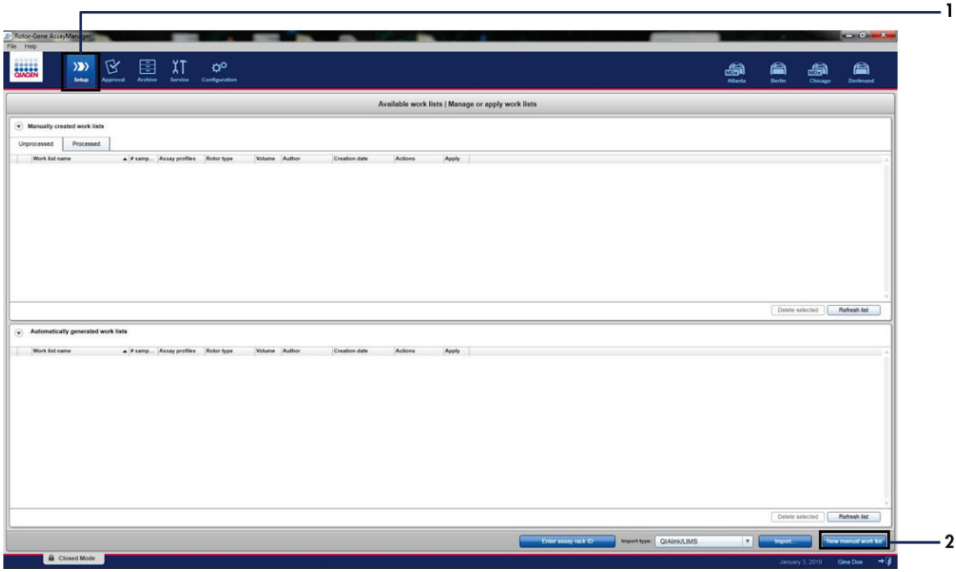


Figure 6. Setting up new manual worklist. 1 = "Setup" tab, 2 = "New manual work list".

15. Select the Assays tab on the left-hand side of the main window. Depending on the sample type, click the **PIK3CA_Tissue_RUO** Assay Profile for tissue samples or **PIK3CA_Plasma_RUO** Assay Profile for plasma samples from the list of available assay profiles, then click the blue arrow to move the assay profile to the Selected assay profiles section. If the assay profile name is truncated, point to the assay profile to see the full name (Figure 7).

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

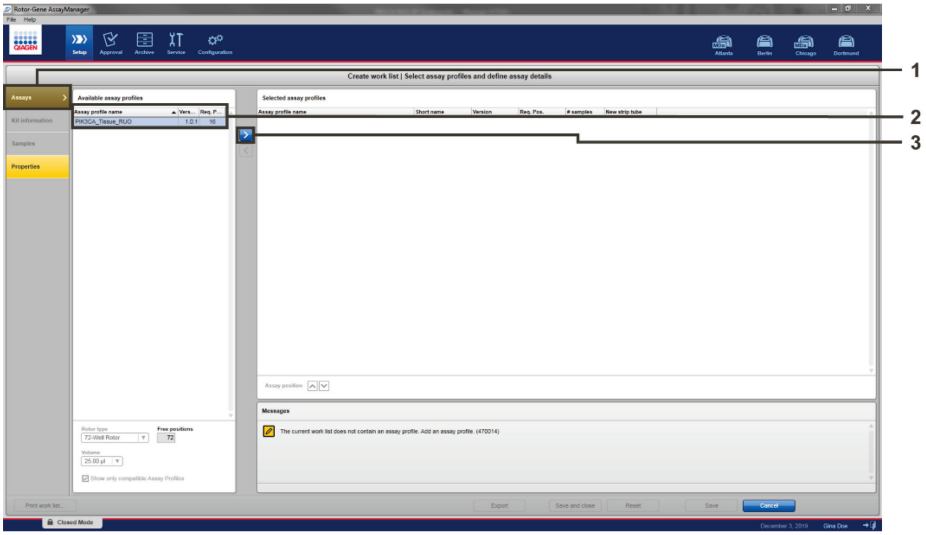


Figure 7. Setting up new manual worklist: Choosing assay profile name. 1 = "Assays" tab, 2 = Available assay profiles with "PIK3CA_Tissue_RUO" or "PIK3CA_Plasma_RUO" selected, 3 = Select the assay profile.

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

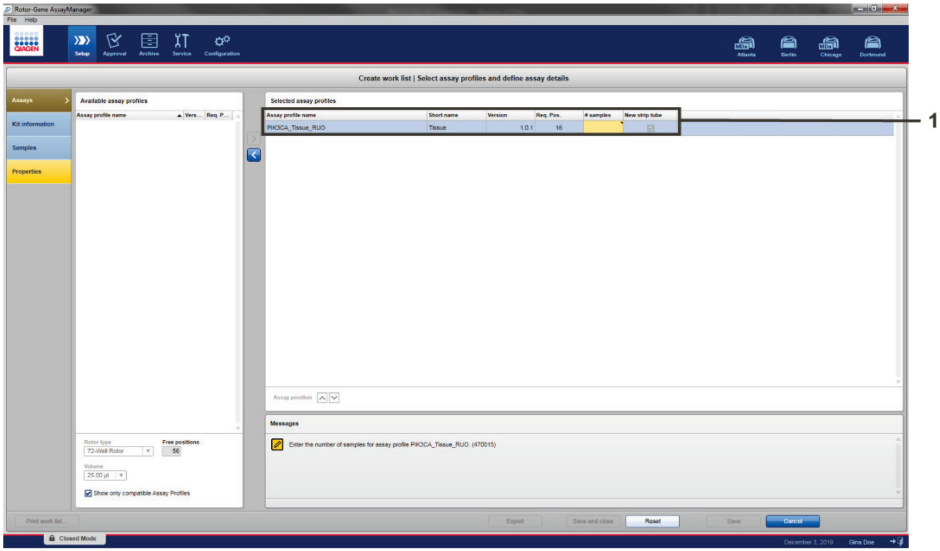


Figure 8. Create work list main window. 1 = Add the number of samples.

17. Click the Kit information tab. Select **Enter kit information manually** and enter the following kit information (Figure 9):
    - Material number
    - Lot number
    - Kit expiry date

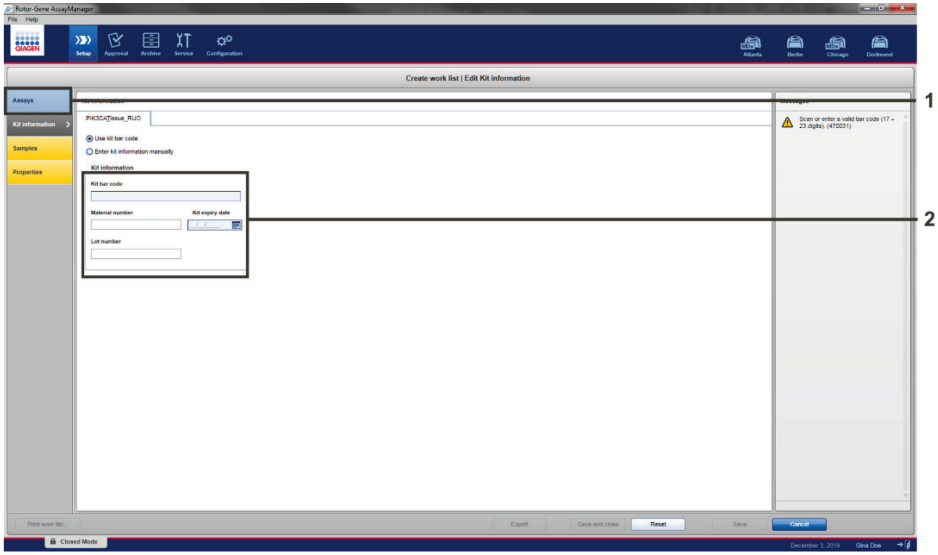


Figure 9. Create work list main window. 1 = "Kit information" tab, 2 = Enter the kit information.

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

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

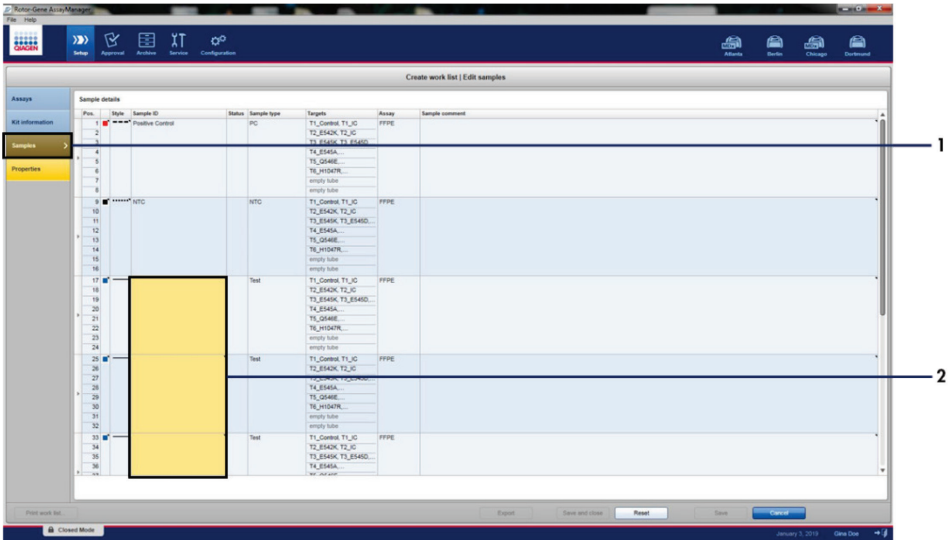


Figure 10. Create work list main window. 1= "Samples" tab, 2 = Entering sample names.

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

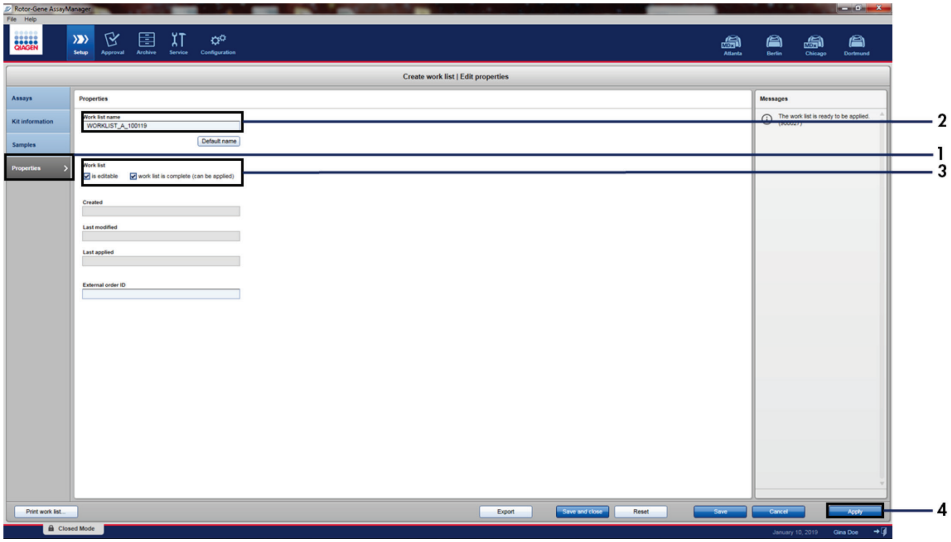


Figure 11. Create work list main window. 1 = "Properties" tab, 2 = Entering worklist name, 3 = Select "is editable" and the "work list is complete", 4 = "Apply".

20. Enter the experiment name in the **Experiment name** field. Select a cycler 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 turns to green to indicate that the run has started.

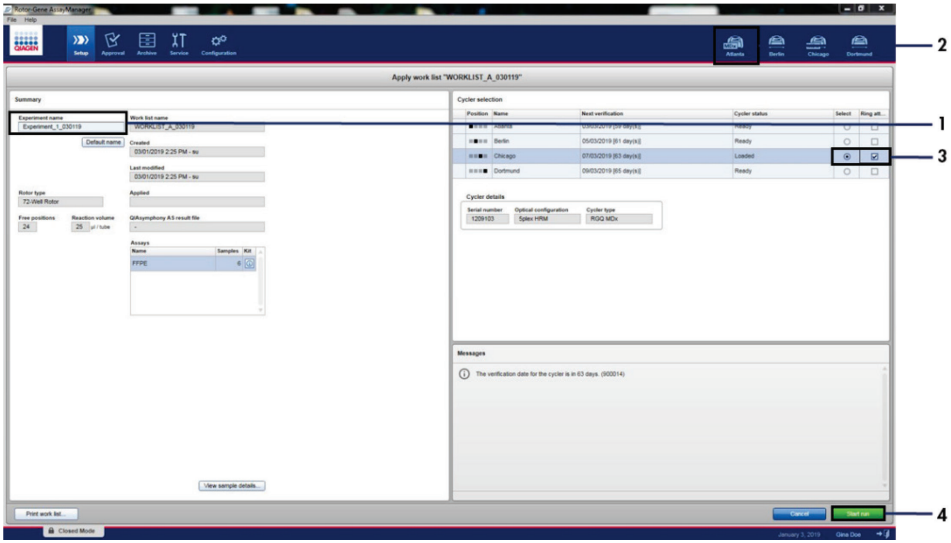


Figure 12. Applying work list and run start. 1 = Enter experiment name, 2 = Instrument selection, 3 = Ensure that "Ring attached" is selected, 4 = Start run.

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

Example cycler icons are shown in Figure 13.

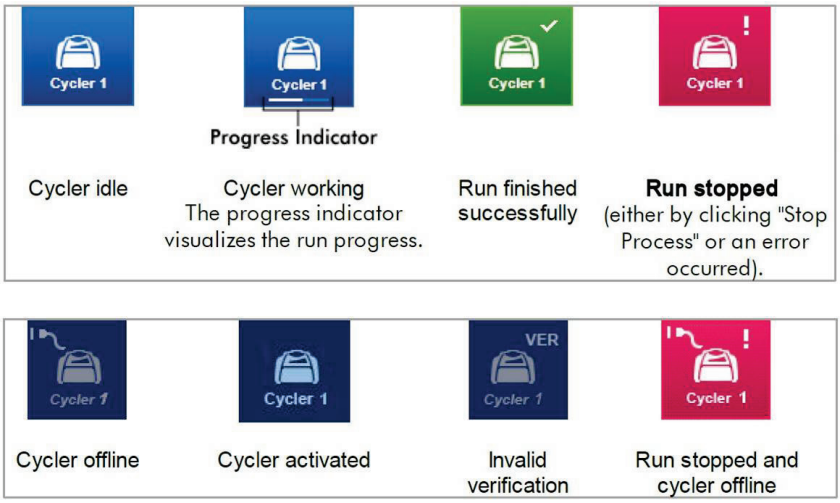


Figure 13. Cycler icons that may be displayed.

21. After the run is complete, click **Finish run**. The Release and go to approval dialog window appears. (Figure 14).

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

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

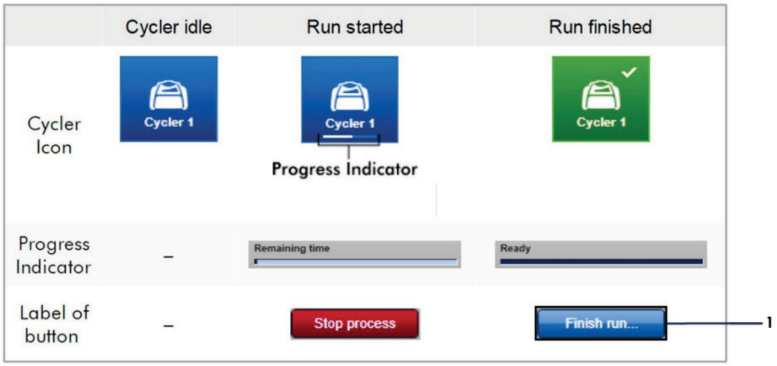


Figure 14. Finishing a run. 1 - "Finish run".

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

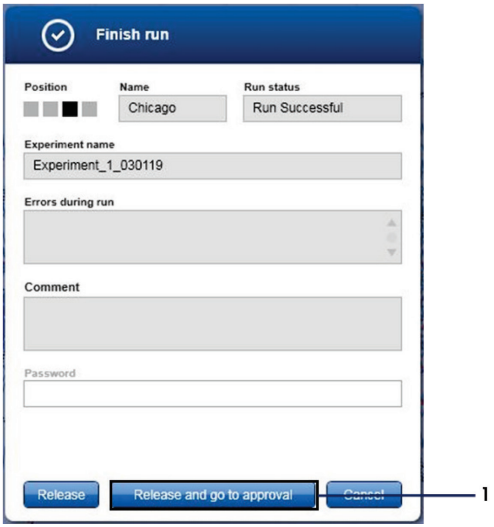


Figure 15. "Finish Run" pop up window. 1 = "Release and go to approval".

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

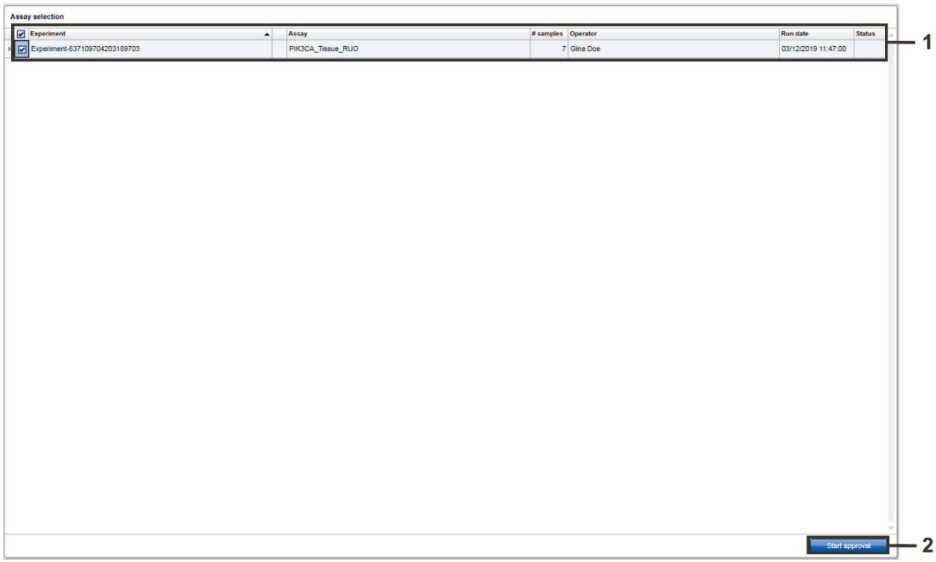


Figure 16. Starting the release process in the "Approval" environment. 1 = Assay selected to approve, 2 = "Start approval".

If the Positive Control and No Template Control are within the QIAGEN-defined acceptable range, the Sample Status column will report **IN_RANGE**; otherwise an **OUT_OF_RANGE** sample status will be reported.

See "Rotor-Gene AssayManager v2.1 PIK3CA Assay Profile flags" for instructions about how to proceed.

Note: The assay profile contains all of the rules for automatic assay data analysis.

24. Click **Release/report data**. The "Release/report data" window appears. (Figure 17).

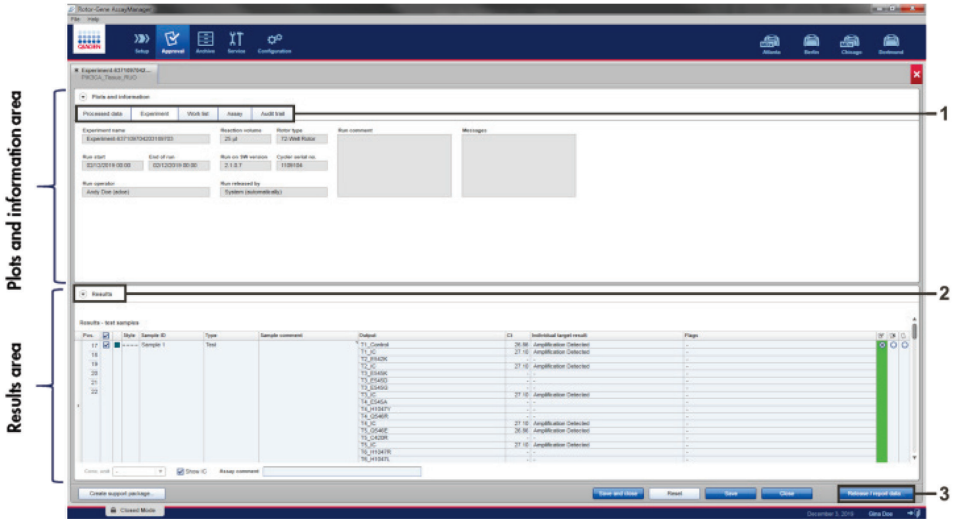


Figure 17. Example of assay results main windows. 1 = "Experiment" tab in the "Plots and information" area. 2 = Results area, 3 = "Release/report data".

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

25. Click **OK** to save the experiment to the archive and create a LIMS output and run report (Figure 18). Run reports and LIMS exports are saved in the default report directory. The default directory can be found in the "Default data export directories" in the Configuration tab.

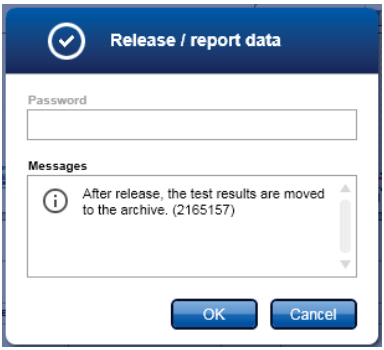


Figure 18. Example of "Release/report data" window.

26. To view an experiment stored in the experiment archive, click **Archive** and search for the experiment using the search criteria in the "Filter Options" section. Click **Apply filter** to search. Select an experiment by checking the box next to the experiment you would like to view, and click **Show assays** (Figure 19).

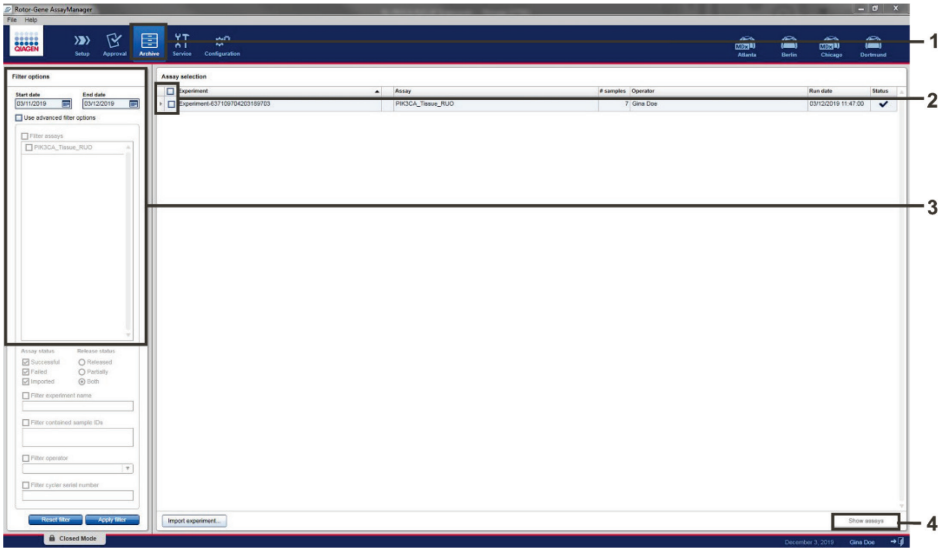


Figure 19. Example of "Experiment Archive" main window. 1 = "Archive" tab, 2 = Search options, 3 = Selecting experiment name, 4 = "Show assays" tab.

Results

Data analysis is performed automatically by the PIK3CA Assay Profile when a run is completed. The following information explains how the PIK3CA Assay Profile carries out the analysis.

Analysis

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

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

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

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

Based on predetermined analytical C_T and ΔC_T values, the PIK3CA Assay Profile qualitatively determines the mutation status of the DNA samples and reports if a mutation is detected.

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

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

All of these assessments are performed automatically and require no manual interpretation. The system automatically checks qPCR data quality and alerts the user if results are outside established normal parameters.

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

The following results may be assigned to an individual sample:

- **Amplification Detected** is displayed if amplification of a target mutation is detected.
- **-** is displayed if no amplification of a target mutation is detected
- **OUT_OF_RANGE**: If one or more data points obtained from analysis of the sample fall outside of the pre-defined expected range which has been set by QIAGEN.

The pre-defined ranges set by QIAGEN on the basis of expected optimal kit performance are presented in Table 6 (PC and NTC Specifications), and Table 7 (IC Assay Specifications). *PIK3CA* Mutation Assay Specifications are presented in the sections 'Performance Characteristics: Tissue Specimens' and 'Performance Characteristics: Plasma Specimens' later in this document.

Table 6. Positive Control (PC) and No Template Control (NTC) Specifications

Assay	Channel	PC C_T acceptable ranges	NTC C_T acceptable ranges
Control	Green Tube 1 and 5	23.39 to 32.39	No amplification detected
E542K	Green Tube 2	22.42 to 31.42	No amplification detected
E545K	Green Tube 3	24.41 to 33.41	No amplification detected
E545D	Yellow Tube 3	23.78 to 32.78	No amplification detected
E545G	Crimson Tube 3	22.61 to 31.61	No amplification detected
E545A	Green Tube 4	22.50 to 31.50	No amplification detected
H1047Y	Yellow Tube 4	26.57 to 35.57	No amplification detected
Q546R	Crimson Tube 4 and 5	24.04 to 33.04	No amplification detected
Q546E	Green Tube 1 and 5	24.72 to 35.72	No amplification detected
C420R	Yellow Tube 5	23.31 to 34.31	No amplification detected
H1047R	Yellow Tube 6	23.33 to 32.33	No amplification detected
H1047L	Crimson Tube 6	24.02 to 33.02	No amplification detected

Table 7. Internal Control Specification

Assay	IC C_T Acceptable range
Internal control (for all tubes and channels, including PC and NTC)	25.51 to 36.51

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

Rotor-Gene AssayManager v2.1 PIK3CA Assay Profile flags

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

Table 8 lists the possible flags that may be generated by the PIK3CA Assay Profiles, their meaning, and actions to be taken.

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

For example:

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

Table 8. 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.	Run can be repeated.
	Invalid sample. IC in sample above the specification range.	The sample can be retested once; if, after retest, the sample IC C_T is still above the acceptable range, re-extract DNA from the original specimen. 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.	Run can be repeated.
(PC)_BELOW_ACCEPTED_RANGE	Invalid run. PC below the specification range.	Run can be repeated.
IC_BELOW_ACCEPTED_RANGE	Invalid run. IC below the specification range in PC or NTC tubes.	Run can be repeated.
	Invalid sample. IC in sample below the specification range	The sample can be retested once; if, after retest, the sample IC C_T is still below the acceptable range, re-extract DNA from the original specimen. If the sample IC is still below the acceptable range after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.
UNEXPECTED_CT_VALUE	Invalid run. C_T value has been detected in NTC.	Run can be repeated.
NO_CT_VALUE	Invalid PC or IC. No C_T value for PC in PC tubes or for IC in PC and NTC tubes.	Run can be repeated.
UNEXPECTED_CT_VALUE	Invalid run. C_T value has been detected in NTC.	Run can be repeated.

Table continued on next page

Table continued from previous page

Flag	Meaning	Action
NO_CT_VALUE	Invalid PC or IC. No C_T value for PC in PC tubes or for IC in PC and NTC tubes.	Run can be repeated.
	Invalid sample. No C_T value in sample.	The sample can be retested once; if, after retest, there is still no sample IC C_T, re-extract DNA from the original specimen. If there is still no sample IC after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.
DNA_INPUT_TOO_HIGH	Invalid sample. Sample Control C_T value below the Control working range.	Sample is too concentrated and must be diluted. Follow instructions in "Control C_T value".
ABOVE_ACCEPTED_RANGE	Invalid sample. Sample Control C_T value above the Control working range.	The sample can be retested once; if, after retest, the Control C_T value is still above the Control working range, re-extract DNA from the original specimen. If the Control C_T value is still above the Control working range after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.
T1_CONTROL_NO_CT_VALUE	Invalid sample. No C_T value for sample in sample control tubes.	The sample can be retested once; if, after retest, the sample has no C_T, re-extract DNA from the original specimen. If the sample still has no C_T after re-extraction and two rounds of testing, then the sample should be reported as indeterminate.

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

Control C_T value

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

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

To calculate the required Control C_T shift (X_R) and estimate the dilution factor required (Table 9):

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

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

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

If X is not a whole number, then round up to the next whole number, e.g., 2.1 is rounded up to 3.0. This value is X_R. Obtain the dilution factor required from Table 9.

Table 9. 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.

- 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 this is not possible, the sample should be reported as indeterminate.

Performance Characteristics: Tissue Specimens

Analytical performance: Tissue specimens

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

Limit of blank (LoB): Tissue specimens

The LoB is defined in CLSI guideline EP17-A2 as "the highest measurement result that is likely to be observed (with a stated probability) for a blank sample". For the PIK3CA RGQ PCR Kit, this is the data point that corresponds to the upper 95% percentile in the mutation-negative samples. The LoB was determined by the analysis of 56 individual clinical wild-type FFPE specimens (30 RES specimens and 26 CNB specimens) tested in duplicate per sample for each of three 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 PIK3CA RGQ PCR Kit were verified to be above the ΔC_T cutoff values determined for each of the assays and are summarized in Table 10 along with the false-positive call rates obtained.

Table 10. Summary of LoB results

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

Limit of detection (LoD): Tissue specimens

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

Table 11. LoD for tissue specimens established using low DNA input samples derived from FFPE clinical specimens and FFPE cell-line specimens

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

MAF: Mutant allele frequency.

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

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

‡ LoD values were established using DNA from clinical specimens.

Genomic DNA input range: Tissue specimens

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

ΔC_T 'in range' values: Tissue specimens

The assay ΔC_T 'in range' value has been determined by QIAGEN to represent a >95% confidence that an "Amplification Detected" result is attributable to the presence of a target *PIK3CA* mutation in a sample. Samples that generate "Amplification Detected" results with a ΔC_T value at or below the cutoff can be regarded as highly likely to contain the target *PIK3CA* mutation. User discretion should be used when determining whether samples that generate "Amplification Detected" results with a ΔC_T value above the cutoff are likely to contain the target *PIK3CA* mutation.

A mixture of cell-line, clinical specimens, and pre-extracted cell-line DNA were used to establish the 'in range' values for each mutation. The values were chosen with respect to the following parameters: false-positive fraction, false-negative fraction, and assay sensitivity.

The 'in range' value for each assay within the PIK3CA RGQ PCR Kit is shown in Table 12.

Table 12. In range values for each mutation assay when testing DNA from tissue specimens

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

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

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

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

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

The PIK3CA RGQ PCR Kit is comprised of six separate reaction mixes: a single Control Reaction that detects a region in exon 15 of the *PIK3CA* gene and 11 mutation assays that detect *PIK3CA* mutations.

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

Interference: Tissue specimens

Effects of necrotic tissue

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

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

Effects of hemoglobin and exogenous substances

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

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

- Paraffin wax
- Xylene
- Ethanol
- Buffer ATL
- Proteinase K
- Buffer AL
- Buffer AW1
- Buffer AW2

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

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

Lot interchangeability: Tissue specimens

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

Specimen handling: Tissue specimens

The reproducibility of the QIAamp DSP DNA FFPE Tissue Kit was examined using sections taken from 11 FFPE specimen blocks; four *PIK3CA* mutant clinical breast cancer specimens, six *PIK3CA* mutant cell-line specimens and one *PIK3CA* wild-type clinical breast cancer

specimen. For each specimen, extractions were carried out in triplicate by two operators, at three sites, yielding a total of 18 data points per specimen. At each site the testing was conducted using one lot of the QIAamp DSP DNA FFPE Tissue Kit and one lot of the PIK3CA RGQ PCR Kit reagents. All valid mutant and wild-type specimen results yielded the expected overall amplification results (correct call = 100%, 18/18 for each specimen). Across specific *PIK3CA* mutation detection PCRs, the proportion of correct amplification detection was 97.92% supporting the reproducibility and repeatability for the PIK3CA RGQ PCR Kit at the pre-analytical step of DNA isolation.

Repeatability and reproducibility: Tissue specimens

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

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

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

For each sample, the proportion of correct calls is shown in Table 13 for repeatability and Table 14 for reproducibility.

Table 13. Assay repeatability – proportion of correct calls for *PIK3CA* mutations tested in DNA samples obtained from FFPE tissue specimens

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

NA: Not applicable.

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

Table 14. Assay reproducibility – proportion of correct calls for *PIK3CA* mutations tested in DNA samples obtained from FFPE tissue specimens

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

NA: Not applicable.

* Samples at LoD with E545A and Q546R *PIK3CA* mutations were evaluated for 6 days across three sites, by three operators, with two runs and four replicates for a total of 144 measurements per site, 432 in total.

A variance component analysis was used to estimate the standard deviation for between-kit, between-run, between-operator, between-instrument, between-day, and within-run variability for repeatability and reproducibility. Across all variance components, the total standard deviation (SD) was ≤1.32 ΔC_T for LoD and ≤0.63 ΔC_T for 3x LoD for all *PIK3CA* mutations tested in the reproducibility testing. Across all mutant panel members, the SD was ≤0.17 ΔC_T for LoD and ≤0.16 ΔC_T for 3x LoD for between-lots (lot interchangeability). The SD for within-run variability (repeatability) was ≤1.24 ΔC_T for LoD and ≤0.53 ΔC_T for 3x LoD.

Cross-contamination/analytical carryover: Tissue specimens

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

This study was performed with H1047R (the mutation with highest prevalence) and wild-type FFPE cell-line specimens. Two independent sets of samples referred to as "Set A" and "Set B" were extracted following a predefined extraction matrix designed to introduce risk of sample cross-contamination. Two operators performed the extractions. A total of 18 extractions (nine per set) were carried out for the mutation positive (H1047R) samples. A total of 42 extractions (21 per set) were carried out for the wild-type samples. The extracts were mutation assessed across 10 PCR runs; five per sample set were set up consecutively by the same operator using the same equipment and Rotor-Gene Q instrument, with no other runs set up using this instrument between these runs. Extracts were tested with the control assay reaction mix (PIK3CA RGQ PCR Kit Tube 1) and mutation of interest (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.

Performance Characteristics: Plasma Specimens

Analytical performance: Plasma specimens

The specific performance characteristics of the 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 PIK3CA RGQ PCR Kit in exons 7, 9, and 20).

Limit of blank (LoB): Plasma specimens

The limit of blank (LoB) is defined in CLSI guideline EP17-A2 as "the highest measurement result that is likely to be observed (with a stated probability) for a blank sample". For the PIK3CA RGQ PCR Kit, this is the data point that corresponds to the upper 95% percentile in the blank samples. To assess performance of the 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 *PIK3CA* wild-type DNA at six input levels were tested in triplicate in a study following guidance from CLSI guideline EP17-A2 to determine the LoB for each mutation assay. All mutation assays gave LoB values above the cutoff for their respective mutations. The LoB of the *PIK3CA* mutants detected by the PIK3CA RGQ PCR Kit from plasma specimens is shown below (Table 15).

Table 15. Summary of LoB results

Reaction mix	Assay	LoB (ΔC_T)	False positive rate (%)
Tube 2	E542K	8.32	0
Tube 3	E545K	15.74	0
	E545D	9.13	0
	E545G	13.39	0
Tube 4	E545A	15.82	0
	H1047Y	9.89	0
	Q546R	10.19	0.56
Tube 5	Q546E	15.82	0
	C420R	11.15	0
Tube 6	H1047R	11.93	0
	H1047L	15.55	0.56

Limit of detection (LoD): Plasma specimens

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

To determine the LoD for each mutation, samples with different percentages of mutation were prepared at low DNA input and tested with the PIK3CA RGQ PCR Kit (Table 16). The LoD for each assay was calculated using a "probit" method. The LoD of 11 contrived mutant samples were established using three different PIK3CA RGQ PCR Kit lots with 24 replicates tested per kit lot per level. A subset of the mutations were verified using clinical plasma samples at the determined LoD.

Table 16. LoD for plasma specimens established using low DNA input clinical and contrived plasma specimens

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

MAF: Mutant allele frequency.

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

† LoD values were established using cell-line specimens.

‡ LoD values were verified using clinical plasma specimens.

Genomic DNA input range: Plasma specimens

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

ΔC_T 'in range' values: Plasma specimens

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

The 'in range' values established are shown in Table 17.

Table 17. In range values for each mutation assay when testing DNA from plasma specimens

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

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

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

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

To assess whether cross-reactivity between mutations detected by the assay has been correctly accounted for in the setting of the 'in range' values, mutant-positive contrived plasma specimens, at high and low DNA input, were diluted to high and low MAF targets and tested in duplicate using three lots of the 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 Amplification detected".

Interference: Plasma specimens

Endogenous substances

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

- Hemoglobin (2 g/l)
- Trigylcerides (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 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 PIK3CA RGQ PCR Kit.

Lot interchangeability: Plasma specimens

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

Specimen handling: Plasma specimens

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

Repeatability and reproducibility: Plasma specimens

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

Repeatability was assessed by testing these samples at one site across 20 non-consecutive days, using three Rotor-Gene Q instruments and by three operators to generate a total of 120 replicates per sample (Table 18).

Table 18. Repeatability overall correct call summary per MAF level

Mutation	Template	Fractional proportion	Percentage	Two-sided lower 95% confidence limit
C402R	LoD	120/120	100.00	96.97
	3x LoD	120/120	100.00	96.97
E542K	LoD	120/120	100.00	96.97
	3x LoD	120/120	100.00	96.97
E542A	LoD	119/120	99.17	95.44
	3x LoD	120/120	100.00	96.97
E545D	LoD	120/120	100.00	96.97
	3x LoD	120/120	100.00	96.97
E545G	LoD	119/120	99.17	95.44
	3x LoD	120/120	100.00	96.97
E545K	LoD*	111/120	92.50	86.24
	3x LoD*	120/120	100.00	96.97
H1047L	LoD	120/120	100.00	96.97
	3x LoD	120/120	100.00	96.97
H1047R	LoD*	110/120	91.67	85.21
	3x LoD*	120/120	100.00	96.97
H1047Y	LoD	120/120	100.00	96.97
	3x LoD	120/120	100.00	96.97
Q546E	LoD	120/120	100.00	96.97
	3x LoD	120/120	100.00	96.97
Q546R	LoD	115/120	95.83	90.54
	3x LoD	120/120	100.00	96.97
WT	C_T30	114/120	95.00	89.43

* For E545K and H1047R the LoD used were 1.99 and 1.44, respectively. The LoD was readjusted, confirmed, and used in a subsequent study. (Table 16).

Reproducibility was measured by testing contrived samples at 1x LoD and 3x LoD level samples across three different sites (one internal QIAGEN site in the United Kingdom and two additional external sites in the USA). All of these samples were tested at each site across 10 non-consecutive days, using three Rotor-Gene Q instruments and by three operators to generate a total of 60 replicates per sample (Table 19).

Table 19. Reproducibility proportion of correct calls across all sites

Mutation	Template	Fractional proportion of valid result	Percentage	Two-sided lower 95% confidence limit
C420R	LoD	237/238	99.58	97.68
	3x LoD	238/238	100.00	98.46
E542K	LOD	237/240	98.75	96.39
	3x LOD	240/240	100.00	98.47
E545A	LOD	239/240	99.58	97.70
	3x LOD	240/240	100.00	98.47
E545D	LOD	240/240	100.00	98.47
	3x LOD	240/240	100.00	98.47
E545G	LOD	237/240	98.75	96.39
	3x LOD	239/239	100.00	98.47
E545K	LOD*	432/432	100.00	99.15
	3x LOD	240/240	100.00	89.47
H1047L	LOD	236/238	99.16	97.00
	3x LOD	238/238	100.00	98.46
H1047R	LOD*	430/432	99.54	98.34
	3x LOD	236/236	100.00	98.45
H1047Y	LOD	239/240	99.58	97.70
	3x LOD	240/240	100.00	98.47
Q546E	LOD	238/238	100.00	98.46
	3x LOD	238/238	100.00	98.46
Q546R	LOD	232/240	96.67	93.54
	3x LOD	240/240	100.00	98.47
WT	C_T30	223/238	93.70	89.82

* Samples at revised LoD with E545K and H1047R (as per Table 16) were evaluated for six days across three sites, by three operators, with two runs and four replicates for a total of 144 measurements per site, 432 in total across all three sites.

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 ≤1.34 ΔC_T for LoD and ≤0.73 ΔC_T for 3x LoD for all *PIK3CA* mutations tested in the reproducibility testing. Across all mutant panel members, the SD was ≤0.20 ΔC_T for LoD and ≤0.10 ΔC_T for 3x LoD for between-lots (lot interchangeability). The SD for within-run variability (repeatability/precision) ranged from 0.415 ΔC_T to 1.407 ΔC_T for LoD and 0.206 ΔC_T to 0.583 ΔC_T for 3x LoD.

Blood collection tube validation

The impact of blood to plasma separation time on plasma specimen quality and subsequent results was determined using contrived blood samples for H1047R, the most prevalent mutation. Whole blood specimens from healthy volunteers were used as wild-type specimens. Blood specimens were collected into 10 ml K_2EDTA tubes from four donors (eight tubes per donor). Contrived blood specimens were generated by spiking *PIK3CA* H1047R mutant fragmented cell-line DNA into blood tubes from two donors after collection. Blood specimens were separated into plasma at approximately 1-, 2-, 3-, and 4-hour time points. DNA was extracted from the plasma specimens using the QIAamp DSP Circulating Nucleic Acid Kit and each target was tested using the PIK3CA RGQ PCR Kit in 16 replicates.

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

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

Troubleshooting Guide

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

		Comments and suggestions
PC sample shows an 'OUT_OF_RANGE' results		
a)	Incorrect configuration of the PCR	Check your pipetting scheme and repeat the PCR.
b)	Error during preparation of the PCR	Repeat the PCR ensuring accurate pipetting.
c)	The storage conditions for one or more kit components did not comply with the instructions given in "Reagent Storage and Handling"	Check the storage conditions (see the kit label) of the reagents and use a new kit, if necessary.
d)	The PIK3CA RGQ PCR Kit has expired	Check the storage conditions and the expiration date (see the kit label) of the reagents and use a new kit, if necessary.
NTC sample shows an 'OUT_OF_RANGE' result		
	Detection of an assay target has occurred in the absence of template material; contamination has occurred during preparation of the PCR	Repetition of the test using sample DNA is recommended, if this is available. If sample DNA is not available, the test may be repeated from the DNA extraction step. If possible, close the PCR tubes directly after addition of the sample to be tested. Make sure that work space and instruments are decontaminated at regular intervals. Consider repeating the test using new reagents.
	Error during preparation of the PCR	Repeat the PCR ensuring accurate pipetting.

Comments and suggestions	
"'DNA input too high" flag in the sample tube	
Sample is too concentrated	Dilute sample to increase C_T value. Samples should be diluted using the water provided in the kit (Water for Dilution [Dil.]).
"Above acceptable range" flag in sample tube	
Insufficient starting DNA template present in sample	**Tissue specimens**: Retest one more time. If system shows the same flag second time, re-extract DNA using two slides from the same specimen of resected tissue or an adequate number of slides for CNBs to obtain 20 mm^2 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". **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". Consider repeating testing with a fresh specimen of blood plasma.
"IC above acceptable range" flag in the sample tube	
Error during preparation of the PCR or inhibitor present in reaction	**Tissue specimens**: Retest one more time. If system shows the same flag a second time, re-extract DNA using two slides from the same specimen of resected tissue or an adequate number of slides for CNBs to obtain 20 mm^2 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". **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 should be recorded as "indeterminate". Consider repeating testing with a fresh specimen of blood plasma.
"No C_T value" flag in T1 Control (sample)	
No amplifiable DNA template present in sample	**Tissue specimens**: Retest one more time. If system shows the same flag second time, re-extract DNA using two slides from the same specimen of resected tissue or an adequate number of slides for CNBs to obtain 20 mm^2 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". **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 should be recorded as "indeterminate". Consider repeating testing with a fresh specimen of blood plasma.

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Contact Information

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Symbols

The following symbols may appear on the packaging and labeling:

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

Ordering Information

Product	Contents	Cat. no.
PIK3CA RGQ PCR Kit (24)	For 24 reactions: 6 Reaction Mixes, Positive Control, *Taq* DNA Polymerase, Water for NTC, and Water for Sample Dilution	873101
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 DNA FFPE Tissue Kit		
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
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
QIAamp DSP Circulating Nucleic Acid Kit		
QIAamp Circulating Nucleic Acid Kit (50)	For 50 DNA preps: QIAamp Mini Columns, Tube Extenders (20 ml), QIAGEN Proteinase K, Carrier RNA, Buffers, VacConnectors, and Collection Tubes (1.5 ml and 2 ml)	55114

Rotor-Gene Q 5plex HRM and accessories

Product	Contents	Cat. no.
Rotor-Gene Q 5plex HRM Platform	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training not included	9001580
Rotor-Gene Q 5plex HRM System	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories: includes 1-year warranty on parts and labor, installation and training	9001650
PCR Tubes, 0.2 ml (1000)	1000 thin-walled tubes for 1000 reactions of 20–50 µl	981005
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Loading Block 72 x 0.1 ml Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 72 x 0.1 ml tubes	9018901
Loading Block 96 x 0.2 ml PCR Tubes	Aluminum block for manual reaction setup with a single-channel pipet in 96 x 0.2 ml PCR tubes	9018905
72-Well Rotor	For holding Strip Tubes and Caps, 0.1 ml, with reaction volumes of 10–50 µl; requires Locking Ring 72-Well Rotor	9018903

Product	Contents	Cat. no.
Locking Ring 72-Well Rotor	For locking Strip Tubes and Caps, 0.1 ml in the 72-Well Rotor	9018904
Rotor Holder	Metal free-standing holder for assembling tubes and Rotor-Discs into rotors	9018908
QIAvac 24 Plus vacuum manifold	For ctDNA purification	19413
QIAvac Connecting System	For ctDNA purification	19419
Vacuum Pump	For ctDNA purification. Or, equivalent pump capable of producing a vacuum of -800 to -900 mbar	84010

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Document Revision History

Date	Changes
December 2019	Initial release
January 2020	Corrected PIK3CA RGQ PCR Kit Plasma Assay Profile version from 1.0.1 to 1.0.0

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