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



Version 1



Qualitative in vitro diagnostics

For use with Rotor-Gene[®] Q MDx



874011



QIAGEN Manchester Ltd Skelton House,
Lloyd Street North, Manchester, M15 6SH, UK



QIAGEN GmbH, QIAGEN Strasse 1,
40724 Hilden, GERMANY



1116068EN

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QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
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Intended Use

The *therascreen*[®] KRAS RGQ PCR Kit is a real-time qualitative PCR assay for the detection of 7 somatic mutations in codons 12 and 13 of the human KRAS oncogene using the Rotor-Gene Q MDx instrument. The kit is intended for use with DNA extracted from formalin-fixed paraffin-embedded (FFPE) samples of colorectal cancer (CRC) or non-small cell lung cancer (NSCLC) samples acquired by resection, core needle biopsy (CNB) or fine needle aspiration (FNA).

Somatic mutations in the KRAS gene are potential predictive biomarkers of resistance to human epidermal growth factor (EGFR)-directed therapies such as panitumumab and cetuximab for the treatment of CRC. Somatic mutations in the KRAS gene may also be indicated as a potential predictive biomarker to make treatment decisions for certain NSCLC therapies.

The patient's mutation status will be considered by a clinician alongside other disease factors to make a therapy decision. No treatment decision for cancer patients should be based on KRAS mutation status alone.

The *therascreen* KRAS RGQ PCR Kit is not intended to diagnose CRC, NSCLC or any other disease.

Summary and Explanation

Mutations in the KRAS oncogene are frequently found in human cancers (1–4). Using Scorpions® and ARMS® (Allele Refractory Mutation System) technologies (5, 6), the *therascreen* KRAS RGQ PCR Kit enables the detection of 7 mutations in codons 12 and 13 of the KRAS oncogene against a background of wild-type genomic DNA (Table 1). Based on data in the COSMIC database (2015 v72), the 7 mutations detected by the *therascreen* KRAS RGQ PCR Kit account for >95% of all reported KRAS mutations in CRC patients and >88% of all reported mutations in NSCLC patients (7).

Table 1. List of mutations and COSMIC identities

Mutation	Base change	COSMIC ID*
GLY12ALA (G12A)	GGT>GCT	522
GLY12ASP (G12D)	GGT>GAT	521
GLY12ARG (G12R)	GGT>CGT	518
GLY12CYS (G12C)	GGT>TGT	516
GLY12SER (G12S)	GGT>AGT	517
GLY12VAL (G12V)	GGT>GTT	520
GLY13ASP (G13D)	GGC>GAC	532

* COSMIC IDs are taken from the *Catalog of Somatic Mutations in Cancer* (7) (www.sanger.ac.uk/genetics/CGP/cosmic).

The test is highly specific and sensitive, enabling detection of a low percentage of mutant DNA against a background of wild-type DNA. If there are sufficient copies of DNA, detection of 0.8% mutant in a background of wild-type genomic DNA is possible (see “Performance Characteristics,” page 51, for limit of detection information for each mutation).

The thescreen KRAS RGQ PCR Kit is used in a polymerase chain reaction (PCR) procedure. The benefit of this kit is that it is highly specific to the target and is fast and efficient with no subjectivity in the determination of the results.

Principle of the Procedure

The *therascreen* KRAS RGQ PCR kit utilizes 2 technologies — ARMS and Scorpions — for detection of mutations in real-time PCR.

Mutation reaction mixes

Each reaction mix uses a mutation-specific ARMS primer to selectively amplify the mutated DNA and then a Scorpions primer to detect the amplification product.

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.

Scorpions

Detection of amplification is performed using Scorpions. Scorpions are bi-functional molecules containing a PCR primer covalently linked to a probe. The probe incorporates the fluorophore carboxyfluorescein (FAM™) and a quencher. The latter quenches the fluorescence of the fluorophore. When the probe binds to the ARMS amplicon during PCR, the fluorophore and quencher become separated, leading to a detectable increase in fluorescence.

Kit format

The *therascreen* KRAS RGQ PCR Kit contains 8 assays:

- 1 control assay (Control Reaction Mix [CTRL])
- 7 mutation assays (12ALA, 12ASP, 12ARG, 12CYS, 12SER, 12VAL, 12ASP)

Reaction mixes are duplex, containing FAM-labeled reagents to detect targets and a HEXTM-labeled internal control. The reaction mixes and positive control reagents contain Tris EDTA buffer, and the positive control contains the carrier Poly A RNA.

Assays

The *therascreen* KRAS RGQ PCR Kit comprises a 2-step procedure. In the first step, the control assay is performed to assess the total amplifiable KRAS DNA in a sample. In the second step, both the mutation and the control assays are performed to determine the presence or absence of mutant DNA.

Control reaction

The CTRL uses a Scorpions primer and an unlabeled primer to amplify a short sequence of exon 4 of the KRAS gene. The control reaction is used to determine if an appropriate level of amplifiable DNA is present in the sample and is a factor used in the analytical calculations that are used to determine mutation status.

Control assay

The control assay, labeled with FAM, is used to assess the total amplifiable KRAS DNA in a sample. The control assay amplifies a region of exon 4 of the KRAS gene. The primers and Scorpion probe are designed to amplify independently of any known KRAS polymorphism.

Mutation assays

Each mutation assay contains a FAM-labeled Scorpion probe and an ARMS primer, for discrimination between wild-type DNA and a specific mutant DNA.

Controls

Note: All experimental runs must contain positive and negative controls.

Internal control

Each reaction mix contains an internal control in addition to the target reaction. A failure indicates that either there may be inhibitors present that could lead to an inaccurate result or an operator setup error has occurred for that tube. If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of the inhibitors. However, it should be noted that this also dilutes the target DNA. A tube of water for sample dilution (Dil.) is provided with the kit. Sample dilution must be performed using the water for sample dilution (Dil.).

Positive control

Each run must contain a positive control in tubes 1–5. The *therascreen* KRAS RGQ Kit contains a KRAS Positive Control (PC) to be used as the template in the positive control reaction. The positive control results are assessed to ensure that the kit performs within the stated acceptance criteria.

Negative control

Each run must contain a negative control (“No Template Control”) in tubes 9–13. The *therascreen* KRAS RGQ PCR Kit contains Water for NTC (NTC) to be used as the “template” for the No Template Control. The No Template Control is used to assess any potential contamination during the run setup and to assess the performance of the internal control reaction.

Sample assessment

The Control Reaction Mix (CTRL) supplied with the *therascreen* KRAS RGQ PCR Kit is used to assess the total amplifiable KRAS DNA in a sample. The control assay amplifies a region of exon 4 of the KRAS gene. It is recommended to set up samples with only the control assay using the KRAS Positive Control (PC) as a positive control and Water for NTC as the No Template Control.

Platform and software

The *therascreen* KRAS RGQ PCR Kit is specifically designed to be used with the Rotor-Gene Q MDx instrument. The Rotor-Gene Q Software and the *therascreen* KRAS Assay Package is available for web download or separately on CD.

Rotor-Gene Q MDx instruments must be maintained according to the requirements in the instrument user manual. Refer to the user manual for information concerning the instrument. See "Appendix 2: Installation of the *therascreen* KRAS Assay Package" for installation instructions.

Materials Provided

Kit contents

<i>therascreen</i> KRAS RGQ PCR Kit					(24)
Catalog no.					874011
Number of preps			Tube identification		24
Color	Identity				Volume
Red	Control Reaction Mix	1	CTRL		2 x 600 µl
Purple	12ALA Reaction Mix	2	12ALA		600 µl
Orange	12ASP Reaction Mix	3	12ASP		600 µl
Pink	12ARG Reaction Mix	4	12ARG		600 µl
Green	12CYS Reaction Mix	5	12CYS		600 µl
Yellow	12SER Reaction Mix	6	12SER		600 µl
Grey	12VAL Reaction Mix	7	12VAL		600 µl
Blue	13ASP Reaction Mix	8	13ASP		600 µl
Beige	KRAS Positive Control	9	PC		250 µl
Mint	Taq DNA Polymerase		<i>Taq</i>		80 µl
White	Water for NTC		NTC		1.9 ml
White	Water for Sample Dilution		Dil.		1.9 ml
<i>therascreen</i> KRAS RGQ PCR Kit Handbook (English)					1

Materials Required but Not Provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

Reagents

- QIAamp® DNA FFPE Tissue Kit (cat. no. 56404; see “DNA extraction”)
- Xylene
- Ethanol (96–100%)*

Consumables

- Sterile pipet tips with filters (to avoid cross-contamination, we recommend pipet tips with aerosol barriers)
- Sterile microcentrifuge tubes for preparing master mixes
- 0.1 ml Strip Tubes and Caps, for use with 72-well rotor (cat. no. 981103 or 981106)

Equipment

- Rotor-Gene Q MDx with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and HEX, respectively)
- Rotor-Gene Q software version 2.3 with the KRAS Assay Package (version 3.1.1) installed for automated mutation detection (see “Appendix 2: Installation of the *therascreen* KRAS Assay Package”).

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

- **Note:** The Rotor-Gene Q software can be used without the KRAS Assay Package for manual mutation detection. See “Appendix 1: *therascreen* KRAS RGQ PCR Kit Manual Protocol.”
- Thermomixer†, heated orbital incubator, heating block or water bath capable of incubation at 56°C and 90°C
- Benchtop centrifuge† with rotor for 1.5 ml tube
- Benchtop vortexer†
- Dedicated pipets (adjustable) for sample preparation*
- Dedicated pipets (adjustable) for PCR master mix preparation*
- Dedicated pipets (adjustable) for dispensing of template DNA*

Warnings and Precautions

For in vitro diagnostic use

Safety information

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.

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

General precautions

The user should always pay attention to the following:

- Store and extract positive materials (specimens and positive controls) separately from all other reagents and add them to the reaction mix in a spatially separated facility.
- Use extreme caution to prevent contamination of PCRs with synthetic control material. We recommend using separate, dedicated pipets for setting up reaction mixes and adding DNA template. Preparation and dispensing of reaction mixes must be carried out in a separate area to the addition of the template. Rotor-Gene Q tubes must not be opened after the PCR run has finished. This is to prevent laboratory contamination with post-PCR products.
- Reagents for the *therascreen* KRAS RGQ PCR Kit have been optimally diluted. We do not recommend further dilution of reagents as this may result in a loss of performance. We do not recommend using reaction volumes of less than 25 µl, since this will increase the risk of false-negative results.
- All reagents in the *therascreen* KRAS RGQ PCR Kit are formulated specifically for optimal performance. All reagents supplied in the kit are intended to be used solely with the other reagents in the same *therascreen* KRAS RGQ PCR Kit. Substitutions to the reagents in the kit must not be made if optimal performance is to be maintained.
- Only use *Taq* DNA polymerase (*Taq*) provided in the kit. Do not substitute with *Taq* DNA polymerase from other kits of the same or any other type, or with *Taq* DNA polymerase from another supplier.

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

Reagent Storage and Handling

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

The *therascreen* KRAS RGQ PCR Kit should be stored immediately upon receipt at -30 to -15°C in a constant-temperature freezer and protected from light. As with all fluorescently labeled molecules, Scorpions must be protected from light to avoid photo bleaching and loss of performance.

When stored under the recommended storage conditions in the original packaging, the *therascreen* KRAS RGQ PCR Kit is stable until the stated expiration date. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 6 freeze–thaw cycles.

Specimen Collection, Preparation for Analysis, and Storage

Note: All samples must be treated as potentially infectious material.

Sample material must be human genomic DNA extracted from FFPE tissue. Specimens must be transported according to standard pathology methodology to ensure specimen quality.

Tumor samples are heterogeneous and data from a sample of tumor may not be concordant with other sections from the same tumor. Tumor samples may also contain non-tumor tissue. DNA from non-tumor tissue would not be expected to contain mutations detected by the *therascreen* KRAS RGQ PCR Kit.

Preparation of tissue samples

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

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

Preparation of tissue samples for DNA extraction (CRC)

- Using standard materials and methods, fix the tissue specimen in 10% neutral buffered formalin (NBF), and embed the tissue specimen in paraffin. Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides.
- A trained individual (e.g., a pathologist) must assess a Hematoxylin & Eosin (H&E)-stained section for tumor content and area determination. Mark the stained slide to distinguish tumor from normal tissue. Use serial sections for DNA extraction.
- Use sections with >20% tumor content by area for processing without macrodissection (see below).

- For sections that are <20% tumor content by area, macrodissect one or more sections. Discard the non-tumor tissue.
- For sections that are <4 mm² in area, process 2 or more sections to increase the total tumor area to at least 4 mm² (applies to samples both with and without macrodissection). Discard the non-tumor tissue.
- Scrape excess paraffin away from the tissue using a fresh, sterile scalpel.

Preparation of tissue samples for DNA extraction (NSCLC)

- Using standard materials and methods, fix the tissue specimen in 10% NBF, and embed the tissue specimen in paraffin. Using a microtome, cut 5 µm serial sections from the paraffin block and mount them on glass slides.
- A trained individual (e.g., a pathologist) must assess a H&E-stained section for tumor presence. Use serial sections for DNA extraction.
- Scrape excess paraffin away from the tissue using a fresh, sterile scalpel.

Storage

Store FFPE blocks and slides at room temperature. Slides may be stored at ambient temperature for up to 4 weeks prior to DNA extraction.

Genomic DNA may be stored at 2–8°C for 1 week post extraction, then at –25 to –15°C up to 8 weeks before use.

Procedure

DNA extraction

The performance characteristics for the *therascreen* KRAS RGQ PCR Kit were generated using DNA extracted with the QIAamp DNA FFPE Tissue Kit (cat. no. 56404). If using the QIAamp DNA FFPE Tissue Kit, carry out the DNA extraction according to instructions in the handbook noting the following.

DNA extraction (CRC samples)

- The QIAamp DNA FFPE Tissue Kit must be used manually only.
- Do not use the RNase step described in the QIAamp DNA FFPE Tissue Kit Handbook.
- Do not use QIAGEN deparaffinization solution. Use only the xylene/ethanol method for deparaffinization, as described in the QIAamp DNA FFPE Tissue Handbook.
- Proteinase K digestion (step 11 in the QIAamp DNA FFPE Tissue Kit Handbook) must be performed for 1 hour.
- The samples must be eluted using 200 µl elution buffer (Buffer ATE) from the QIAamp DNA FFPE Tissue Kit.

DNA extraction (NSCLC samples)

- Use 2 x 5 µm sections per extraction.
- The QIAamp DNA FFPE Tissue Kit must be used manually only.
- Do not use the RNase step in the QIAamp DNA FFPE Tissue Kit Handbook.
- Do not use the QIAGEN deparaffinization solution provided in the QIAamp DNA FFPE Tissue Kit. Use only the xylene/ethanol method for deparaffinization described in the QIAamp DNA FFPE Tissue Kit Handbook.
- Proteinase K digestion (step 11 in the QIAamp DNA FFPE Tissue Kit Handbook) must be performed for 1 hour.
- Add 60 µl elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit and incubate for 2.5 minutes at room temperature.
- Centrifuge at full speed for 1 minute.
- Add a further 60 µl elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit and incubate for 2.5 minutes at room temperature.
- Centrifuge at full speed for 1 minute.

Protocol: DNA sample assessment

This protocol is used to assess the total amplifiable DNA in samples using the KRAS CE Sample Assessment Locked Template (Assay Package) for automated sample assessment.

Note: For manual sample assessment, refer to “Appendix 1: *therascreen* KRAS RGQ PCR Kit Manual Protocol.”

Important points before starting

- Up to 24 samples can be assessed using the CTRL available.
- Use the CTRL to assess the DNA prior to testing.

- **Note:** It is important to use the CTRL as described below for this assessment and not spectrophotometry or other alternative methods. Heavily degraded DNA may not amplify even though the primers generate short DNA fragments.
- For efficient use of the reagents in the *therascreen* KRAS RGQ PCR Kit, batch DNA samples as far as possible to create full runs. Testing samples individually or in smaller numbers uses up more reagents and reduces the overall number of samples that can be tested with a single *therascreen* KRAS RGQ PCR Kit.
- Ensure that the correct *therascreen* KRAS Assay Package software corresponding to the Rotor-Gene Q software version is installed before first use of the Rotor-Gene Q MDx instrument (see “Appendix 2: Installation of the *therascreen* KRAS Assay Package”).

Procedure

1. Completely thaw the control reaction mix (tube CTRL), nuclease-free water for No Template Control (NTC), and KRAS Positive Control (PC) at room temperature (15–30°C) for a minimum of 1 hour.

Note: Bring Taq DNA polymerase (Taq) to room temperature (15–30°C) at the same time as the other reagents (see “Reagent Storage and Handling”). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

The times for thawing reagents, PCR setup, and storage before starting the run are indicated in Table 2.

Note: Perform PCR setup at room temperature.

Table 2. Thawing time, PCR setup times, and storage temperatures

Minimum	Thaw time		Storage* temperature after PCR setup	Maximum PCR setup and storage time
	Minimum	Maximum		
1 hour	1 hour	4.5 hours	Room temperature (15-30°C)	7 hours
1 hour	1 hour	4.5 hours	2–8°C	18 hours

* “Storage” refers to the time between completion of PCR setup and start of the PCR run on the Rotor-Gene Q MDx instrument.

1. **Mix the thawed reagents by inverting each tube 10 times to avoid localized concentrations of salts, then centrifuge briefly to collect the contents at the bottom of the tube.**

Note: Do not vortex the *Taq* DNA polymerase (*Taq*) or any mix containing *Taq*, as this may inactivate the enzyme.

2. **Prepare sufficient master mixes (Control Reaction Mix [CTRL] plus *Taq* DNA polymerase [*Taq*]) according to the volumes in Table 3 for the following:**

- All the DNA samples
- 1 KRAS Positive Control (PC) reaction
- 1 nuclease-free water for No Template Control (NTC) reaction
- 1 extra sample to allow sufficient overage for the PCR setup

The master mix contains all of the components needed for the PCR, except the sample.

Table 3. Preparation of Control assay master mix

Component	Volume
Control Reaction Mix (CTRL)	19.76 μl x (n+1)*
<i>Taq</i> DNA polymerase (<i>Taq</i>)	0.24 μl x (n+1)*
Total volume	20 μl/reaction

* n = Number of reactions (samples plus controls).

Prepare enough master mix for an extra sample (n+1) to allow for sufficient overage for the PCR setup.

The value n should not exceed 24 (plus controls) as 24 is the maximum number of samples which can fit on a run.

Note: When preparing the master mix, the required volume of the Control Reaction Mix (CTRL) is added to the relevant tube first and the *Taq* DNA polymerase (*Taq*) is added last.

Note: Pipet *Taq* DNA polymerase by carefully placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.

- Place the appropriate number of PCR 4-strip tubes (each strip has 4 tubes) in the loading block according to the layout in Table 4. Do not cap the tubes.

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

Table 4. Run layout in the loading block for DNA sample assessment

Assay									
Control	1 (PC)	9	17	25	–	–	–	–	–
Control	2 (NTC)	10	18	26	–	–	–	–	–
Control	3	11	19	–	–	–	–	–	–
Control	4	12	20	–	–	–	–	–	–
Control	5	13	21	–	–	–	–	–	–
Control	6	14	22	–	–	–	–	–	–
Control	7	15	23	–	–	–	–	–	–
Control	8	16	24	–	–	–	–	–	–

* Numbers denote positions in the loading block and indicate final rotor position.

- Set a pipet to a volume lower than the total volume of the reaction master mix and mix thoroughly by fully aspirating up and down 10 times.

- Immediately add 20 µl master mix to each PCR strip tube.

Note: See Table 4 for the tube layout. For DNA sample assessment, Control assay master mix should be added to one PC tube, one NTC tube, and one tube for each DNA sample.

- Immediately add 5 µl of nuclease-free water for No Template Control (NTC) to the NTC tube (tube position 2) and cap the tube.

- Add 5 µl of each DNA sample to the sample tubes (tube positions 3–26) and cap the tubes.

8. **Add 5 μ l of KRAS Positive Control (PC) to the PC tube (tube position 1) and cap the tube.**
Each tube should contain a total reaction volume of 25 μ l (20 μ l master mix prepared in Table 3, plus 5 μ l of NTC/sample/PC).
9. **Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor Gene Q MDx instrument.**
10. **Invert capped tubes 4 times to mix the sample and reaction mix.**
11. **Place all PCR 4 strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 4) using marks for orientation.**
Note: If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q MDx instrument is maintained.
12. **Place the 72-well rotor into the Rotor-Gene Q MDx instrument. Make sure the locking ring (supplied with the Rotor-Gene Q MDx instrument) is placed on top of the rotor to secure the tubes during the run.**
13. **Start the Rotor-Gene Q software and open the template at the same time by double-clicking the “therascreen KRAS QC Locked Template” icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument (Figure 1).**

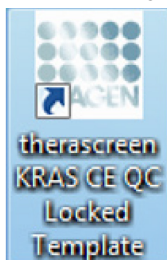


Figure 1. The “therascreen KRAS QC Locked Template” icon.

14. **The “Setup” tab appears as the default (Figure 2). Ensure that the locking ring is properly attached and check the “Locking Ring Attached” box. Close the lid of the Rotor-Gene Q MDx instrument.**

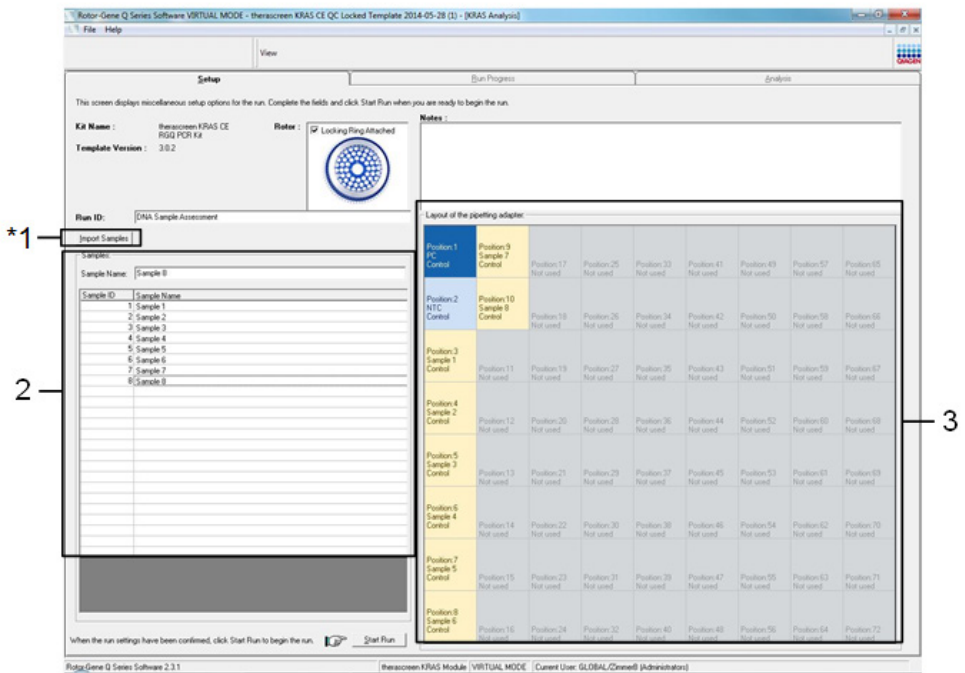


Figure 2. The “Setup” tab and “Locking Ring Attached” box. 1 = “Setup” tab, 2 = “Locking Ring Attached” box.

15. Enter the run ID in the “Run ID” dialog field according to your local naming convention. Enter the sample name in the “Sample Name” dialog field according to your local naming convention and press the return key.

This will add the sample name to the sample list below and assign the sample a “Sample ID” (1, 2, 3, etc.). In addition, the “Layout of the pipetting adapter” panel on the right side will update to include the sample name (Figure 3).

Alternatively, sample names stored in the *.smp (Rotor-Gene Q sample file) or *.csv (comma separated values) format can be imported using the “Import Samples” button. Sample names will be populated automatically using this method.

Note: In the “Layout of the pipetting adapter” panel, check that addition of the sample name has been highlighted by a change in color and that the sample name is in the sample position (Figure 3).

Note: Sample names with more than 8 characters may not be completely displayed in the “Layout of the pipetting adapter” panel.

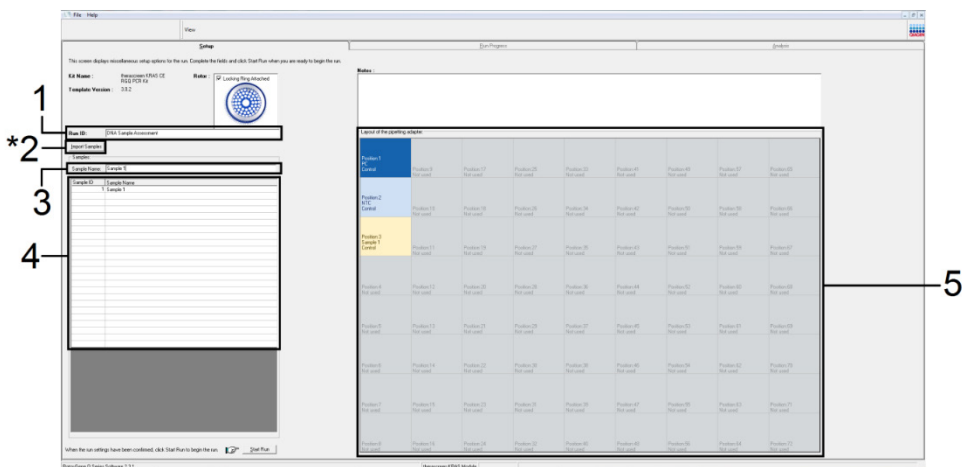


Figure 3. Entering the “Run ID” and “Sample Name.” 1 = “Run ID” dialog field, 2 = “Import Sample” button 3 = “Sample Name” dialog field, 4 = Sample List, 5 = “Layout of the pipetting adapter” panel.

16. Repeat step 16 to enter the names of all additional samples (Figure 4).

Note: To edit a sample name, click on the “Sample Name” in the sample list and the selected sample will appear in the “Sample Name” dialog field above. Edit the sample name according to your local naming convention and press the return key to update the name.

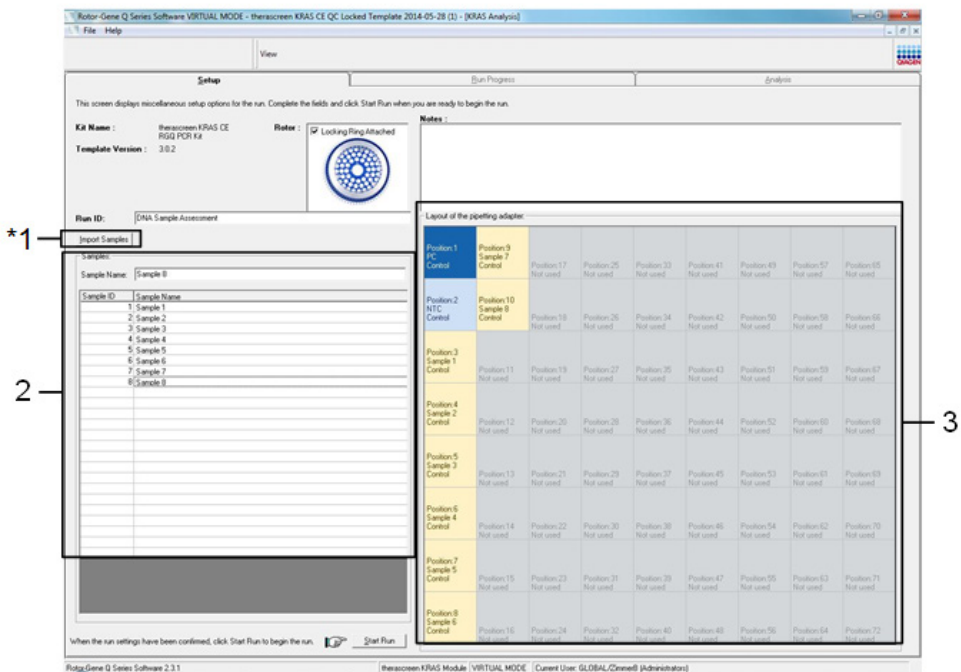


Figure 4. Entering additional sample names in the “Sample Name” dialog field. *1 = “Import Sample” button, 2 = “Sample Name” dialog field and Sample List, 3 = “Layout of the pipetting adapter” panel with additional sample name.

17. When all sample names have been entered, verify they are correct. Add any additional information in the “Notes” dialog field if necessary and then click “Start Run” (Figure 5).

Note: If any rotor position is unused, a “Warning” will appear (Figure 5 and Figure 6) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube. Check that all unused rotor positions are filled with a capped, empty tube and click “OK” to proceed.

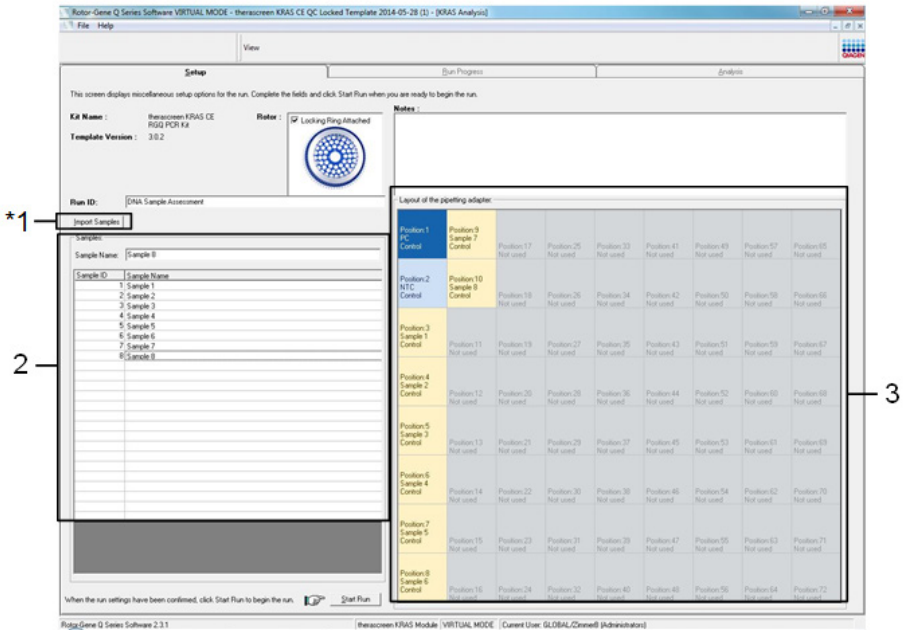


Figure 5. "Notes" dialog field, "Start Run," and "Warning" of unused rotor positions.

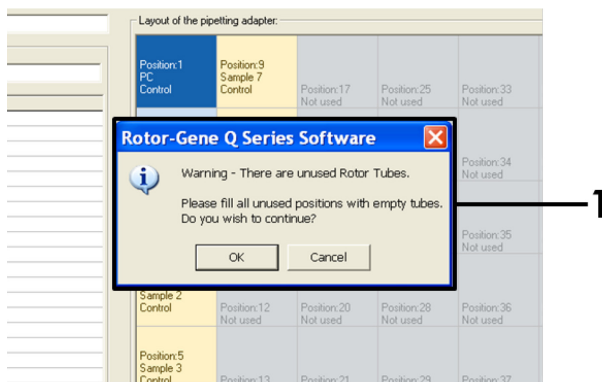


Figure 6. 1 = "Warning" of unused rotor positions.

18.A “Save As” window appears. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location. Click “Save” (Figure 7).

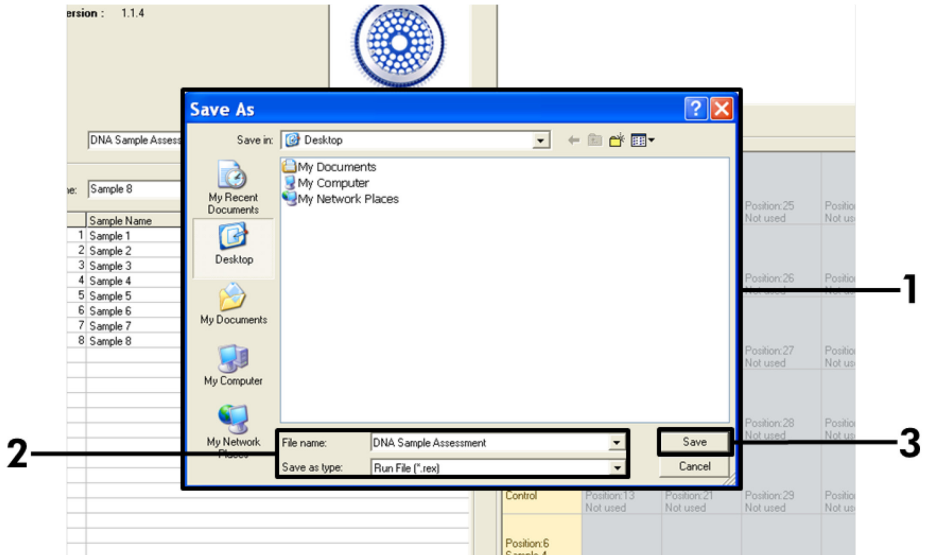


Figure 7. Saving the run file. 1 = “Save As” window, 2 = File name and save as type *.rex file, 3 = “Save.”

19. The PCR run starts.

Note: When the run starts, the “Run Progress” tab will open automatically to show the temperature trace and remaining run time (Figure 8).

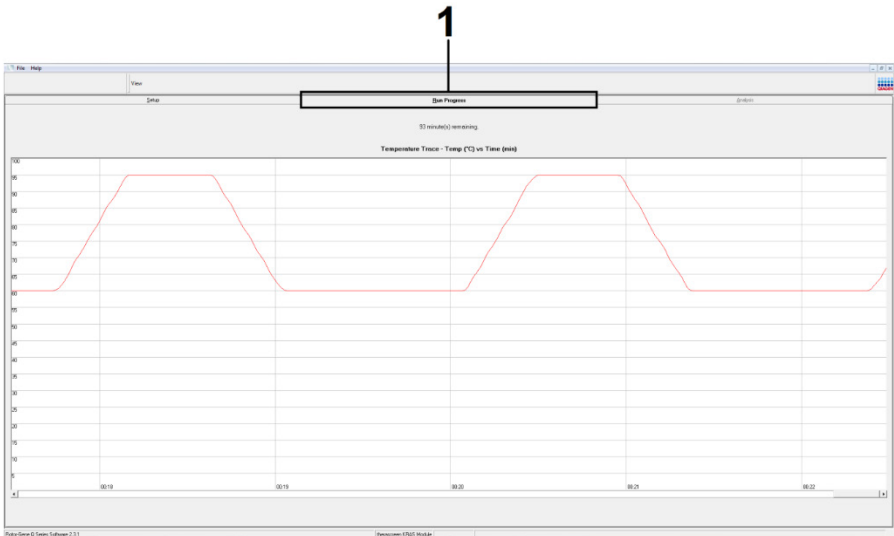


Figure 8. The Run Progress tab.

20. After the run is finished, the “Analysis” tab will open automatically.

Note: If the “Analysis” tab fails to open, click the “Analysis” tab (Figure 9).

Note: An explanation of the calculation method is presented in “Interpretation of Results” section.

The screenshot shows a software window with a menu bar (File, Help, View) and a toolbar (Setup, Run Progress, Analysis, Report). The 'Analysis' tab is active. A table titled 'Sample QC Result Table' is displayed, containing the following data:

Tube ID	Sample Name	Control Assay Ct	Flags/Warnings	Status
1	PC Control	25.50	-	Valid
2	NTC Control	-	-	Valid
3	037710706	29.39	-	Valid
4	037710718	27.38	-	Valid
5	037710728	30.07	-	Valid
6	037710738	26.53	-	Valid
7	037710748	29.95	-	Valid
8	037710758	29.45	-	Valid
9	037710768	29.95	-	Valid
10	037710778	29.02	-	Valid
11	037710788	31.42	-	Valid
12	037710798	29.93	-	Valid
13	037710818	29.60	-	Valid
14	037710828	31.44	-	Valid
15	037710838	31.02	-	Valid
16	037710848	28.09	-	Valid
17	037710858	29.91	-	Valid
18	037710878	30.33	-	Valid
19	037710888	30.22	-	Valid
20	037710898	27.17	-	Valid
21	037710908	29.87	-	Valid
22	037710918	29.32	-	Valid
23	037710928	28.22	-	Valid
24	037710938	28.57	-	Valid
25	037710948	29.88	-	Valid
26	037710958	30.41	-	Valid

Figure 9. The “Analysis” tab and reporting of results. 1 = “Analysis” tab, 2 = “Sample QC Result Table.”

21. Control results will be reported as follows in the “Sample QC Result Table” (2 in Figure 9).

- **Run controls (PC and NTC, tube positions 1 and 2, respectively):** “Valid” is displayed if results are within acceptable ranges. Otherwise, an “Invalid” result will appear.
- **Sample control reaction $C_T > 32.00$:** “Invalid” is displayed. Quantity of DNA is not sufficient for mutation analysis. Retest the sample. If the quantity of DNA is still insufficient, extract more tumor tissue if available (see “Troubleshooting guide”).
- **Sample control reaction $C_T < 21.92$:** “Invalid” is displayed. DNA concentration is too high for mutation analysis. Dilute with Nuclease- Free Water for Dilution (Dil.) and retest. Dilute to a C_T of 21.92–32.00. A 1:1 dilution increases the C_T value by approximately 1.0.
- **Sample control reaction C_T of 21.92–32.00** ($21.92 \leq \text{Control } C_T \leq 32.00$): “Valid” is displayed DNA concentration is suitable for mutation analysis.

Note: If re-extraction or dilution is required, repeat the control reaction to confirm that the DNA concentration is suitable for use.

22. Report files can be produced by clicking “Report.” The “Report Browser” window will appear. Select “KRAS Analysis Report” under “Templates” and then click “Show” (Figure 10).

Note: Reports can be saved to an alternative location in Web Archives format by clicking the “Save As” button on the top left corner of each report.

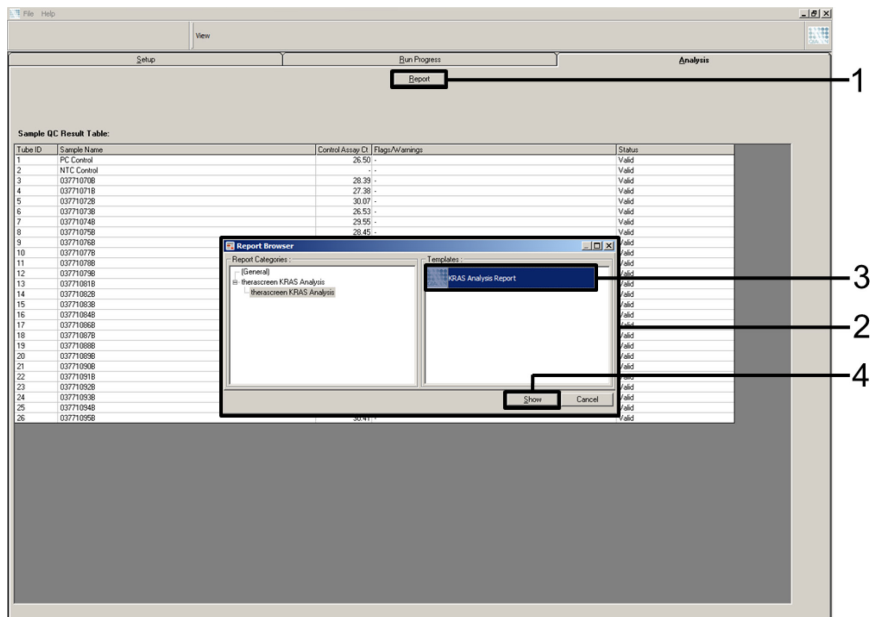


Figure 10. Selecting the “KRAS Analysis Report.” 1 = “Report,” 2 = “Report Browser” window, 3 = “KRAS Analysis Report” selection, 4 = “Show.”

Protocol: Detection of KRAS mutations

This protocol is for the detection of KRAS mutations.

Important points before starting

- A sample can be tested using the KRAS mutation assays once it has passed the sample assessment.
- For efficient use of the *therascreen* KRAS RGQ PCR Kit, samples must be grouped into batches of 7 (to fill the 72-well rotor). Smaller batch sizes will mean that fewer samples can be tested with the *therascreen* KRAS RGQ PCR Kit.
- Ensure that the correct *therascreen* KRAS Assay Package software corresponding to the Rotor-Gene Q software version is installed before first use of the Rotor-Gene Q MDx instrument (see “Appendix 2: Installation of the *therascreen* KRAS Assay Package”).

Procedure

1. **Mix the thawed reagents by inverting each tube 10 times to avoid localized concentrations of salts. Centrifuge briefly to collect the contents at the bottom of the tube.**
2. **Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the master mixes by fully aspirating up and down 10 times.**
3. **Immediately add 20 µl master mix to each appropriate PCR strip tube.**

Note: See Table 5 for the tube layout while setting up the reaction mixes. For detection of KRAS mutations, master mixes should be added to 8 PC tubes, 8 NTC tubes and 8 tubes for each DNA sample.

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

Assay	Controls		Sample number						
	PC	NTC	1	2	3	4	5	6	7
CTRL	1*	9	17	25	33	41	49	57	65
12ALA	2	10	18	26	34	42	50	58	66
12ASP	3	11	19	27	35	43	51	59	67
12ARG	4	12	20	28	36	44	52	60	68
12CYS	5	13	21	29	37	45	53	61	69
12SER	6	14	22	30	38	46	54	62	70
12VAL	7	15	23	31	39	47	55	63	71
13ASP	8	16	24	32	40	48	56	64	72

* Numbers denote positions in the loading block and indicate final rotor position.

4. Immediately add 5 µl of nuclease-free water for No Template Control (NTC) to the NTC tubes (tube positions 9–16) and cap the tubes.
5. Add 5 µl of each DNA sample to the sample tubes (tube positions 17–72) and cap the tubes.
6. Add 5 µl of KRAS Positive Control (PC) to the PC tubes (tube positions 1–8) and cap the tubes.
7. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g. positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q MDx instrument.
8. Invert the capped tubes 4 times to mix the sample and reaction mix.
9. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 5) using the marks for orientation.

Note: A maximum of 7 samples can be included in each PCR run. If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q MDx instrument is maintained.

10. Place the 72-well rotor into the Rotor-Gene Q MDx instrument. Ensure the locking ring (supplied with the Rotor-Gene Q MDx instrument) is placed on top of the rotor to secure the tubes during the run.
11. Start the Rotor-Gene Q software and open the template at the same time by double-clicking on the “therascreen KRAS Locked Template” icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument (Figure 11).



Figure 11. The “therascreen KRAS Locked Template” icon.

12. The “Setup” tab appears as the default (Figure 12). Ensure that the locking ring is properly attached and check the “Locking Ring Attached” box. Close the lid of the Rotor-Gene Q MDx instrument.

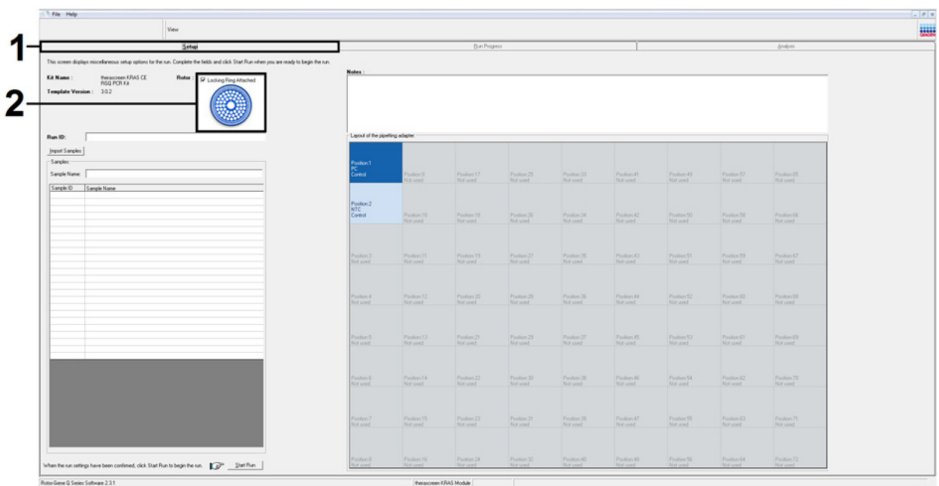


Figure 12. 1 = The “Setup” tab and 2 = “Locking Ring Attached” box.

13. Enter the run ID in the “Run ID” dialog field according to your local naming convention.

14. Enter the sample name in the “Sample Name” dialog field according to your local naming convention and press the return key.

This will add the sample name to the sample list below and assign the sample a “Sample ID” (1, 2, 3, etc.). In addition, the “Layout of the pipetting adapter” panel on the right side will update to include the sample name (Figure 13).

Note: In the “Layout of the pipetting adapter” panel, check that addition of the sample name has been highlighted by a change in color and that all 8 assays in the column under the sample circle are highlighted (Figure 13).

Note: A maximum of 7 samples can be added. The sample IDs (in the sample circles) will automatically be assigned from 1 to 7.

Note: Sample names with more than 8 characters may not be completely displayed in the “Layout of the pipetting adapter” panel.

Alternatively, sample names stored in the *.smp (Rotor-Gene Q sample file) or *.csv (comma separated values) format can be imported using the “Import Samples” button. Sample names will be populated automatically using this method.

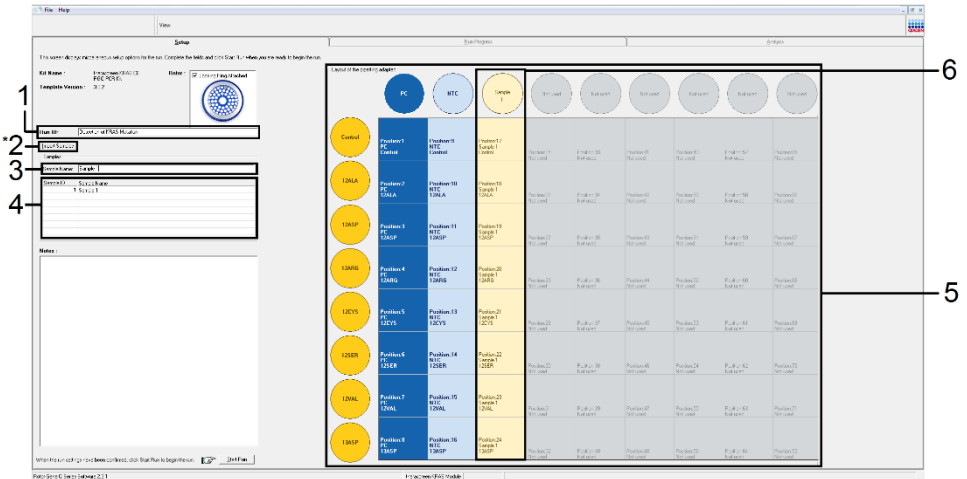


Figure 13. Entering the “Run ID” and “Sample Name.” 1 = “Run ID” dialog field, 2 = “Import Sample” (not available with software version 2.1), 3 = “Sample Name” dialog field, 4 = Sample List, 5 = “Layout of the pipetting adapter” panel, 6 = Highlighted sample circle and column of 8 assays underneath.

15. Repeat step 14 to enter the names of all additional samples (Figure 14).

Note: To edit a sample name, click on the “Sample Name” in the sample list and the selected sample will appear in the “Sample Name” dialog field above. Edit the sample name according to your local naming convention and press the return key to update the name.

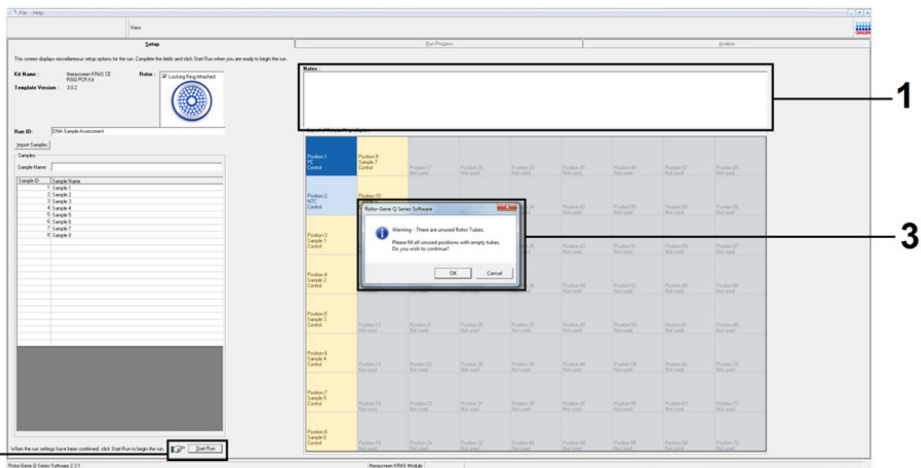


Figure 14. Entering additional sample names in the “Sample Name” dialog field. 1 = “Sample Name” dialog field, 2 = Sample List, 3 = “Layout of the pipetting adapter” panel with additional sample names.

16. When all sample names have been entered, verify they are correct. Add any additional information in the “Notes” dialog field if necessary and then click the “Start Run” button (Figure 15).

Note: If any rotor position is unused, a “Warning” will appear (Figure 15 and Figure 16) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube. Check that all unused rotor positions are filled with a capped, empty tube and click “OK” to proceed.

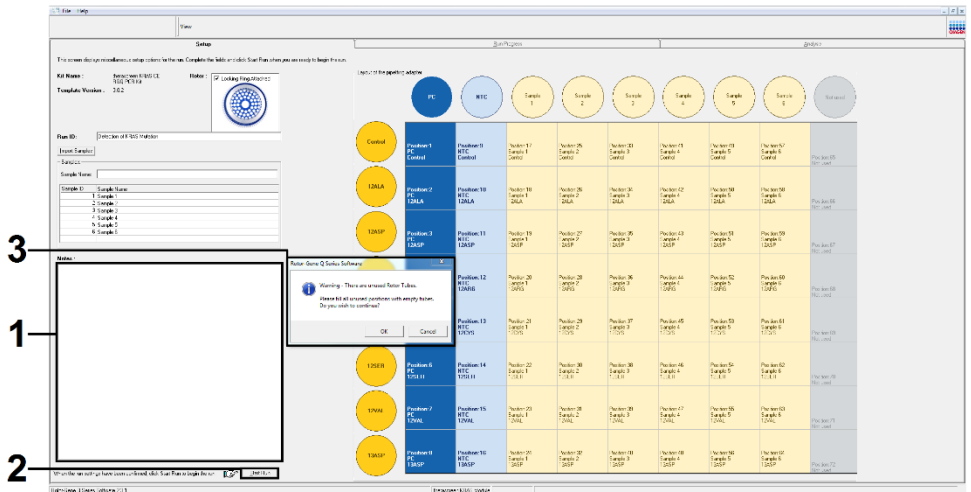


Figure 15. 1 = "Notes" dialog field, 2 = "Start Run," and 3 = "Warning" of unused rotor positions.

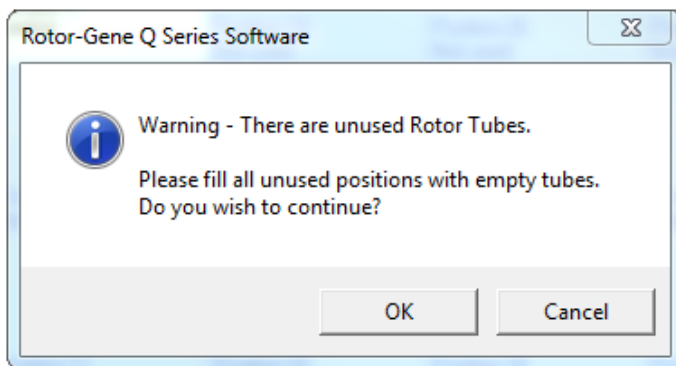


Figure 16. "Warning" of unused rotor positions.

17.A “Save As” window appears. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location (Figure 17).

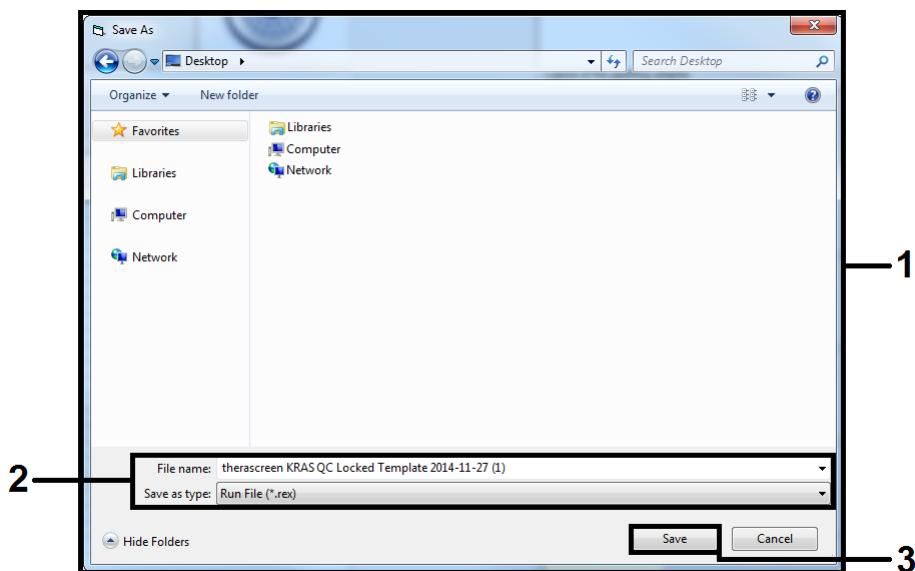


Figure 17. Saving the run file.

18. The PCR run starts.

Note: When the run starts, the “Run Progress” tab will open automatically to show the temperature trace and remaining run time (Figure 18).

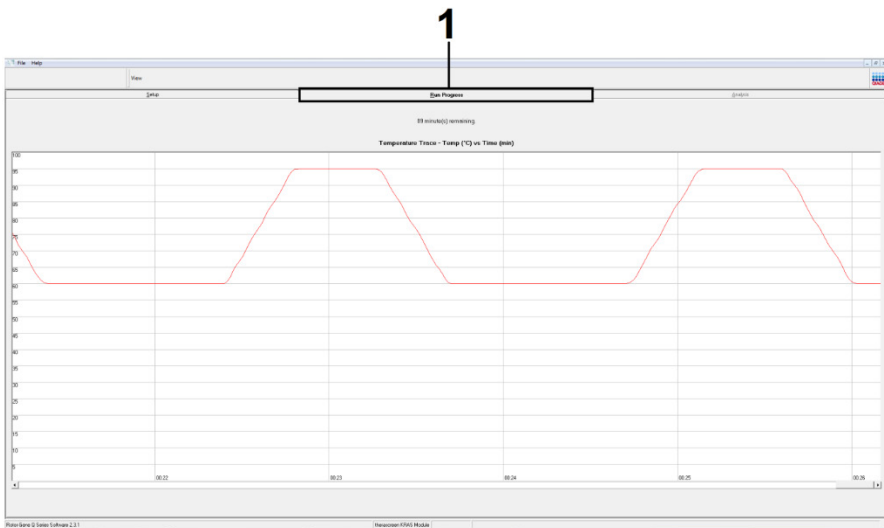


Figure 18. "Run Progress" tab.

19. After the run is finished, the "Analysis" tab will open automatically.

Note: If the "Analysis" tab fails to open, click the "Analysis" tab (Figure 19).

Note: An explanation of the calculation method is presented in the "Interpretation of Results" section.

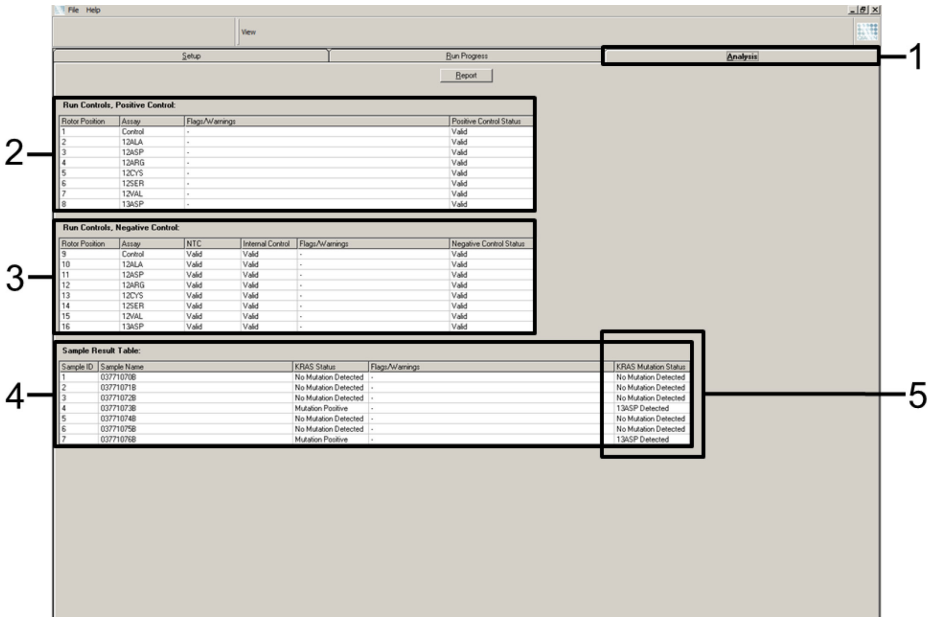


Figure 19. The “Analysis” tab and reporting of results. 1 = “Analysis” tab, 2 = “Run Controls, Positive Control” panel, 3 = “Run Controls, Negative Control” panel, 4 = “Sample Result Table,” 5 = “KRAS Mutation Status” column.

20. Assay results will be reported as follows (Figure 19).

- **The “Run Controls, Positive Control” panel:** If the results are within acceptable range, the “Positive Control Status” will display “Valid,” otherwise an “Invalid” result will appear.
- **The “Run Controls, Negative Control” panel:** If both the “NTC” and “Internal Control” results are within acceptable ranges, the “Negative Control Status” will display “Valid,” otherwise an “Invalid” result will appear.
- **The “Sample Result Table” panel:** Specific mutations will be reported for the Mutation Positive samples under the “KRAS Mutation Status” column.

21. Report files can be produced by clicking “Report.” The “Report Browser” window will appear. Select “KRAS Analysis Report” under “Templates,” and then click “Show” (Figure 20).

Note: Reports can be saved to an alternative location in Web Archives format by clicking “Save As” on the top left corner of each report.

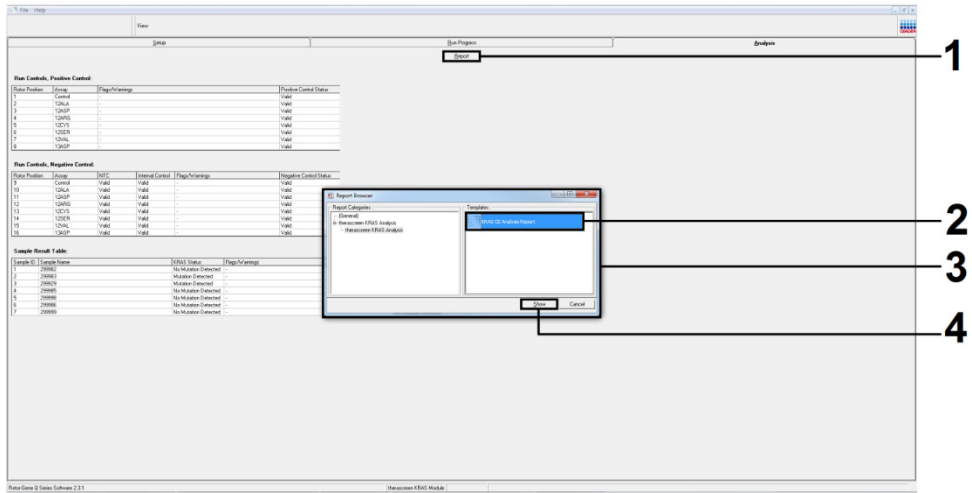


Figure 20. Selecting the “KRAS Analysis Report.” 1 = “Report,” 2 = “Report Browser” window, 3 = “KRAS Analysis Report” selection, 4 = “Show.”

Interpretation of Results

The analysis and mutation calls are performed automatically by the *therascreen* KRAS Assay Package once a run is completed. The following information explains how the *therascreen* KRAS Assay Package makes the analysis and mutation calls.

Note: For manual analysis, refer to “Appendix 1: *therascreen* KRAS RGQ PCR Kit Manual Protocol.”

The PCR cycle at which the fluorescence from a particular reaction crosses a threshold value is defined as the C_T value. C_T values indicate the quantity of specific input DNA. Low C_T values indicate higher input DNA levels and high C_T values indicate lower input DNA levels. Reactions with a C_T value are classed as positive amplification.

The Rotor-Gene Q software interpolates fluorescence signals between any 2 recorded values. The C_T values can therefore be any real number (not limited to integers) within the range of 0 to 40.

For the *therascreen* KRAS RGQ PCR Kit, the threshold value is set at 0.05 relative fluorescence units. This value is configured in the *therascreen* KRAS Assay Package for both the fluorescence channels for Green and Yellow. The threshold value was defined during development of the *therascreen* KRAS RGQ PCR Kit.

A calculation is performed to determine the ΔC_T value using the equation:

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

The run controls (positive control, NTC, and internal controls) are assessed to ensure that acceptable C_T values are met, and the reactions are performing correctly.

Sample ΔC_T values are calculated as the difference between the mutation assay C_T and control assay C_T from the same sample. Samples are classed as mutation positive if they give a ΔC_T less than or equal to the cutoff ΔC_T value for that assay. Above this value, the sample may either contain less than the percentage of mutation able to be detected by the *therascreen* KRAS RGQ PCR Kit (beyond the limit of the assays), or the sample is mutation negative, which would be reported as “No Mutation Detected.”

No amplification in mutation reactions will be scored as “No Mutation Detected.” ΔC_T values calculated from background amplification are expected to be greater than the cutoff ΔC_T values, and the sample will be classed as “No Mutation Detected.”

The assay results will be displayed as “Mutation Positive,” “No Mutation Detected,” “Invalid,” or, if a run control fails, “Run Control Failed.” For the mutation-positive samples, specific mutations will be reported.

Other possible results that may be displayed are discussed in the “Protocol: DNA sample assessment” of this handbook.

Rarely, a tumor may contain more than one mutation. In such instances, the mutation yielding the lowest ΔC_T value will be identified.

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 either the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit www.qiagen.com).

Comments and suggestions

Invalid results

- | | |
|--|--|
| a) The storage conditions for one or more components did not comply with the instructions given in "Reagent Storage and Handling." | Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit, if necessary. |
| b) The <i>therascreen</i> KRAS RGQ PCR Kit has expired. | Check the storage conditions and the expiration date (see the kit label) of the reagents and, if necessary, use a new Kit. |

NTC samples show positive results in the FAM channel.

- | | |
|---|---|
| Contamination occurred during preparation of the PCR. | Repeat the PCR with new reagents in replicates.
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. |
|---|---|

Flags generated by the *therascreen* KRAS Assay Package

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

Table 6. theascreen KRAS Assay Package flags

Flag	Meaning	Action to be taken
PC_CTRL_ASSAY_FAIL	PCR run invalid — FAM CT out of range for positive control in control reaction.	Repeat the entire PCR run.
PC_MUTATION_ASSAY_FAIL	PCR run invalid — FAM CT out of range for one or more mutation control reactions.	Repeat the entire PCR run.
PC_CTRL_INVALID_DATA	PCR run invalid — Fluorescence data in positive control (Control Reaction Mix) can't be interpreted.	Repeat the entire PCR run.
PC_MUTATION_INVALID_DATA	PCR run invalid — Fluorescence data in positive control (mutation reaction mix) can't be interpreted.	Repeat the entire PCR run.
NTC_INT_CTRL_FAIL	PCR run invalid — Internal control above range for negative control.	Repeat the entire PCR run.
NTC_INT_CTRL_EARLY_CT	PCR run invalid — Internal control is below range for negative control.	Repeat the entire PCR run.
NTC_INVALID_CT	PCR run invalid — FAM invalid (smaller than limit) for negative control.	Repeat the entire PCR run.
NTC_INVALID_DATA	PCR run invalid — Fluorescence data in negative control can't be interpreted.	Repeat the entire PCR run.
SAMPLE_CTRL_INVALID_DATA	Sample invalid — Fluorescence data in sample control can't be interpreted.	Setup new PCR run to repeat the relevant sample(s).
SAMPLE_CTRL_HIGH_CONC	Sample Invalid — FAM C _T too low in sample control.	Dilute sample to increase control C _T value. This dilution should be calculated on the assumption that diluting 1:1 with the water supplied in the kit will increase the C _T by 1.0; once sample is diluted, setup new PCR run to repeat sample.
SAMPLE_CTRL_FAIL	Sample invalid — FAM C _T too high in sample control reaction.	Set up new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Set up a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

SAMPLE_INT_CTRL_FAIL	C _T too high (or no C _T) for internal control (HEX), FAM channel mutation-negative.	<p>If sample given valid status — no action.</p> <p>CRC samples: If sample given invalid status, set up new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Set up a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.</p> <p>NSCLC samples: If sample given invalid status, dilute the remaining sample 1 in 8 with water from the tube marked DIL ensuring the final volume is greater than 40 µl (e.g., 10 µl of DNA and 70 µl of water from the tube marked DIL), set up a new PCR run to repeat the sample. If invalid on the repeat PCR run, extract the sample from a fresh FFPE section(s). Set up a new PCR run to test fresh extraction. If invalid, dilute the remaining sample 1 in 8 with water from the tube marked DIL ensuring the final volume is greater than 40 µl, then test this dilution. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.</p>
SAMPLE_INT_CTRL_EARLY_CT	Mutation tube invalid — C _T HEX too low for sample (internal control)	<p>If sample given valid status — no action.</p> <p>If sample given invalid status, set up new PCR run to repeat sample.</p> <p>If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Set up a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.</p>
SAMPLE_INVALID_DATA	Mutation tube invalid — Fluorescence data in internal control can't be interpreted.	<p>If sample given valid status — no action.</p>

If sample given invalid status, set up new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Set up a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

MUTATION_EARLY_CT

Mutation tube invalid — C_T FAM too low for sample.

If sample given valid status — no action.
If sample given invalid status, set up new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Set up a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

SAMPLE_POSITIVE
_AND_INVALID

One or more mutations for a sample are valid and positive; at the same time, one or more mutations for the same sample are invalid (warning, not an error).

None.

Quality Control

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

Limitations

The test is designed to detect 7 mutations in codons 12 and 13 of the KRAS gene. Samples with results reported as "No Mutation Detected" may harbor KRAS mutations not detected by the assay (e.g., 13CYS).

Detection of mutations is dependent on sample integrity and the amount of amplifiable DNA present in the specimen. The procedure should be repeated in the event that the initial assessment of the DNA in the sample indicates that the quantity is either not sufficient or too high for mutation analysis.

The *therascreen* KRAS RGQ PCR Kit is used in a polymerase chain reaction (PCR) procedure. As with all PCR procedures, samples may be 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 reaction mix reagents.

The *therascreen* KRAS RGQ PCR Kit is only intended to discriminate between wild-type and mutant. The test has been designed such that each mutant reaction is most sensitive for the specific mutation being measured. However, in samples where a mutation is detected, cross-reactivity may occur with other mutation reactions. If more than one mutant reaction is positive, the result is the one with the lowest ΔC_T result.

The *therascreen* KRAS RGQ PCR Kit is only validated for FFPE CRC and NSCLC tissue.

The *therascreen* KRAS RGQ PCR Kit is only validated for use with the QIAamp DNA FFPE Tissue Kit. Only the Rotor-Gene Q MDx has been validated for use with the *therascreen* KRAS RGQ PCR Kit.

Performance Characteristics

Analytical performance

The specific performance characteristics of the *therascreen* KRAS RGQ PCR Kit were determined by studies involving FFPE tissue samples collected from CRC patients and NSCLC patients. Acquisition methods for NSCLC samples included core needle biopsy (CNB), fine needle aspirate (FNA), and resection. For each sample type, 8 FFPE human cell lines, of which 7 harbor known KRAS mutations detected by the assay and one KRAS wild-type (i.e., no mutations at codon 12 and 13) were used. Mutation status of samples was confirmed by bi-directional Sanger sequencing.

Cutoff

Using a method following guidance in CLSI EP17-A (2004) (8), 225 FFPE samples were tested to establish the cutoffs for the assay. The control reaction C_T range was established as 21.92 to 32.00. The cutoff values, which are based on the C_T of the control reaction subtracted from the C_T of the mutant reactions (ΔC_T) are shown in Table 7.

Table 7 Established cutoff values for each mutation assay.

	Mutation assay						
	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
Cutoff ($\leq \Delta C_T$)	8.0	6.6	8.0	8.0	8.0	7.5	7.5

Limit of blank

To assess performance of the *therascreen* KRAS RGQ PCR Kit in the absence of mutant positive template, and to ensure that a blank sample does not generate an analytical signal that may indicate a low concentration of mutation, samples with no template were evaluated. The results demonstrated no detectable control or mutant C_T values in any of the mutation or control reaction tubes (internal control C_T values were all valid).

Comparison to analytical reference method: CRC

Two studies were conducted to demonstrate the concordance in mutation status of CRC samples tested with the *therascreen* KRAS RGQ PCR Kit relative to bi-directional sequencing. A total of 137 of the FFPE samples returned valid results for both the *therascreen* KRAS RGQ PCR Kit and bi-directional sequencing.

The overall results, excluding 6 failed bi-directional Sanger sequencing samples, are shown in Table 8. Table 9 shows the analysis of agreement between the *therascreen* KRAS RGQ PCR Kit and bi-directional sequencing.

Table 8. *therascreen* KRAS RGQ PCR Kit vs. bi-directional Sanger sequencing

		Mutation call by bi-directional sequencing							Total	
		Neg.	12ALA	12ARG	12ASP	12CYS	12SER	12VAL		13ASP
<i>therascreen</i> KRAS RGQ PCR Kit call	Negative	80	–	–	1	–	–	–	1	82
	Positive 12ALA	–	3	–	–	–	–	–	–	3
	Positive 12ARG	–	–	–	1	–	–	–	–	1
	Positive 12ASP	–	–	–	20	–	–	–	–	20
	Positive 12CYS	–	–	–	–	3	–	–	–	3
	Positive 12SER	–	–	–	–	–	–	–	–	0
	Positive 12VAL	2	–	–	–	–	–	14	–	16
	Positive 13ASP	1	–	–	–	–	–	–	11	12
	Total	83	3	0	22	3	0	14	12	137

Table 9. Analysis of agreement

Measurement of agreement	Frequency (%)	95% Confidence Interval (CI)
Overall percentage agreement	132/137 (96.35)	92.69–98.21
Percentage positive agreement	52/54 (96.30)	89.41–98.77
Percentage negative agreement	80/83 (96.39)	91.30–98.55

A second unique set of samples was evaluated to supplement the data from the first study. A set of 271 CRC FFPE samples was procured; 250 of unknown mutation status and 21 samples of known mutation status (to enrich for rare mutations) were compared to Sanger bi-directional sequencing, as described above.

Concordance analysis was carried out on 247 samples with both valid bi-directional and *therascreen* KRAS RGQ PCR Kit results. There were 9 discordant samples. Overall, agreement

was 96.4%. The data support the accurate performance of the *therascreen* KRAS RGQ PCR Kit (Table 10 and Table 11).

Table 10. *therascreen* KRAS RGQ PCR Kit vs. bi-directional Sanger sequencing (second study)

		Mutation call by bi-directional sequencing								
		Neg.	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP	Total
<i>therascreen</i> KRAS RGQ PCR Kit call	Negative	132	–	–	–	–	1	–	–	133
	Positive 12ALA	–	10	–	–	–	–	–	–	10
	Positive 12ARG	5	–	5	–	–	–	–	–	10
	Positive 12ASP	–	–	–	31	–	–	–	–	31
	Positive 12CYS	1	–	–	–	11	–	–	–	12
	Positive 12SER	–	–	–	–	–	13	–	–	13
	Positive 12VAL	2	–	–	–	–	–	25	–	27
	Positive 13ASP	–	–	–	–	–	–	–	11	11
	Total	140	10	5	31	11	14	25	11	247

Table 11. Analysis of agreement (second study)

Measurement of agreement	Frequency (%)	95% Confidence Interval (CI)
Overall percentage agreement	238/247 (96.36)	93.73–98.09
Percentage positive agreement	106/107 (99.07)	95.64–99.95
Percentage negative agreement	132/140 (94.29)	89.93–97.13

Comparison to analytical reference method: NSCLC

To demonstrate the concordance in mutation status of NSCLC samples tested with the *therascreen* KRAS RGQ PCR Kit when compared with bi-directional Sanger sequencing, clinical FFPE NSCLC samples acquired via resection, CNB or FNA. DNA was extracted from each sample prior to testing with the *therascreen* KRAS RGQ PCR Kit. The results from this test were compared to those obtained via bi-directional Sanger sequencing.

A total of 360 samples returned a valid result for both the *therascreen* KRAS RGQ PCR Kit and bi-directional Sanger sequencing, with 340 samples having concordant results.

Agreement between the *therascreen* KRAS RGQ PCR Kit and bi-directional sequencing is shown in Table 12. Two samples returned double mutation calls by bi-directional Sanger sequencing. Since one mutation was the same as the *therascreen* KRAS RGQ PCR Kit result, these samples were classed as concordant for the overall agreement, positive agreement and negative agreement analysis (Table 13).

Table 12. *therascreen* KRAS RGQ PCR Kit vs. bi-directional Sanger sequencing

		Mutation call by bi-directional sequencing								
		Neg.	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP	Total
<i>therascreen</i> KRAS RGQ PCR Kit call	Negative	132	–	–	–	–	1	–	–	133
	Positive 12ALA	–	10	–	–	–	–	–	–	10
	Positive 12ALA_12CYS	5	–	5	–	–	–	–	–	10
	Positive 12ARG	–	–	–	31	–	–	–	–	31
	Positive 12ASP	–	–	–	–	–	–	–	–	–
	Positive 12CYS	1	–	–	–	11	–	–	–	12
	Positive 12SER	–	–	–	–	–	13	–	–	13
	Positive 12VAL	2	–	–	–	–	–	25	–	27
	Positive 13ASP	–	–	–	–	–	–	–	11	11
	Total	140	10	5	31	11	14	25	11	247

Table 13. Analysis of agreement

Measurement of agreement	Frequency (%)	95% Confidence Interval (CI)
Overall percentage agreement	340/360 (94.44)	92.03–96.29
Percentage positive agreement	79/80 (98.75)	94.21–99.94
Percentage negative agreement	261/280 (93.21)	90.20–95.51

Limit of detection (LOD)

The working range for the *therascreen* KRAS RGQ PCR Kit is based on the amount of amplifiable DNA in the specimen as determined by the control reaction C_T value. The stated input range for the assay is defined by the control C_T pre-specified range of 21.92 to 32.00. The LOD is the minimum percentage of mutant DNA that can be detected in a background of wild-type when the total amplifiable DNA is within the stated input range and still below the threshold cutoff ΔC_T value.

CRC

A study was conducted to determine the LOD of each of the 7 mutation-specific reactions incorporated in the *therascreen* KRAS RGQ PCR Kit. For the *therascreen* KRAS RGQ PCR Kit, the limit of detecting mutant DNA in a background of wild-type DNA is defined as the lowest dilution factor at which 95% of the test replicates for each mutation positive sample were determined to be positive.

Logistic regression models were applied to each assay individually for the low- and high-input DNA datasets. In these models, the response variable was the binary output of mutation detected (detect = 1) and mutation not detected (detect = 0), the continuous explanatory variable was \log_2 % mutation dilution. The LODs were calculated as the percent mutation dilution that gave a predicted probability of detection of 0.95 (Table 14).

Table 14. LOD values for each mutation assay using FFPE cell lines

Assay	LOD C₉₅ (percentage of mutant DNA in wild-type DNA)
12ALA	0.8
12ARG	2.6
12ASP	6.4
12CYS	1.5
12SER	5.6
12VAL	1.6
13ASP	6.4

NSCLC

The LOD for the *therascreen* KRAS RGQ PCR Kit assays was determined and verified using CRC tissue. These LOD results have been re-verified for NSCLC tissue.

The study was in 2 parts. In Part 1, 60 replicates of 7 mutant FFPE NSCLC cell lines representing each mutation were diluted to the LOD of the respective assay and tested. All 60 valid FFPE cell line replicates for each sample assessed demonstrated 100% detection for their respective mutation reaction at the LOD assessed.

In Part 2, 96 replicates of clinical FFPE NSCLC samples, representing each mutation across 3 acquisition methods (resection, CNB and FNA), were tested after dilution to the LOD of the respective assay.

The 96 valid replicates for 12ALA, 12ASP, 12ARG, 12VAL and 13ASP showed 100% correct call. The assays for 12CYS and 12SER showed 95.8% detection at the LOD.

This demonstrates that the previously determined LOD value is verified for all mutation assays when assessing NSCLC tissue and clinical FFPE NSCLC/FFPE cell lines/patient-matched samples.

DNA input and linearity

Effect of DNA input level on ΔC_T values

When samples at different total DNA levels contain the same proportion of mutant DNA, it is expected that the measured ΔC_T values will remain consistent. DNA extracted from 8 FFPE cell lines was used to prepare pools of DNA with the lowest achievable control reaction C_T .

The dilution range for each mutation reaction and the mean ΔC_T value obtained from the results are shown in Table 15 and Table 16. Overall ΔC_T values are consistent across the working range of the *therascreen* KRAS RGQ PCR Kit for all assays, demonstrating that the DNA level will not impact on the accuracy of sample mutation calling.

Table 15. Effect of DNA input on ΔC_T values across the input control reaction C_T range — CRC FFPE cell lines

Assay	ΔC_T				
	Dilution 1 ~20–21 C_T	Dilution 2 ~23–24 C_T	Dilution 3 ~26–27 C_T	Dilution 4 ~29–30 C_T	Dilution 5 ~32–33 C_T
12ALA	1.56	1.25	1.16	1.14	1.27
12ASP*	2.46	2.18	2.11	2.11	1.75
12ARG	1.18	0.63	1.08	0.94	1.06
12VAL	0.29	0.25	0.15	0.26	-0.1
12SER	2.91	2.21	2.15	2.15	2.08
12CYS	0.98	0.71	0.58	0.81	0.67
13ASP	3.57	2.84	2.54	2.46	2.62

* The total number of replicates for 12ASP was 27.

Table 16. Effect of DNA input on ΔC_T values across the input control reaction C_T range — NSCLC FFPE samples

Assay	ΔC_T				
	Dilution 1 ~20–21 C_T	Dilution 2 ~23–24 C_T	Dilution 3 ~26–27 C_T	Dilution 4 ~29–30 C_T	Dilution 5 ~32–33 C_T
12ALA	3.40	3.25	3.11	2.90	3.31
12ASP	3.63	2.92	2.55	2.46	–*
12ARG	2.49	2.22	2.25	2.23	1.40
12VAL	1.34	1.23	1.18	1.13	0.97
12SER	5.34	4.50	4.30	3.92	–*
12CYS	1.70	1.71	1.70	1.77	1.01
13ASP	6.24	5.36	5.14	4.87	–*

* No mutation reaction C_T returned due to low concentration of DNA, therefore no ΔC_T was calculated.

Linearity/amplification efficiency as a function of DNA input

The linearity and amplification efficiency of PCR for each mutation reaction, relative to the Control Reaction, across the working range of the *therascreen* KRAS RGQ PCR Kit was demonstrated. Amplification efficiency was calculated for each of the mutation reactions and the control reaction as $[2(-1/\text{slope})] - 1$.

The amplification efficiency of the control compared to the mutant reaction indicates that the ΔC_T , and thus mutation call, is consistent across the working range of the assay. A summary of the data is shown in Table 17 and Table 18.

Linearity/amplification efficiency as a function of percent mutation

The objective of this study was to evaluate the effect of serially diluted mutant-positive sample on amplification efficiency, across the working range of the *therascreen* KRAS RGQ PCR Kit, beginning with input levels of C_T at approximately 22–23 C_T .

DNA extracts from CRC FFPE cell lines and NSCLC samples were initially assessed by OD readings prior to carrying out PCR with the *therascreen* KRAS RGQ PCR Kit. DNA stocks were then prepared to a Control Reaction C_T corresponding to approximately $23C_T$. The stocks were diluted serially two-fold each time using wild-type DNA, in order to maintain the total wild-type DNA constant while varying the percentage mutant DNA in the template.

Pools of DNA sufficient for 6 replicates per mutation were prepared. C_T and ΔC_T data for each mutation at each dilution point were calculated. A linear regression model was fitted with mutation reaction C_T versus \log_2 DNA input dilution. The study showed the dilution of mutations in a background of a constant concentration of wild-type DNA resulted in amplification efficiencies that did not vary significantly outside the values determined in the above linearity study.

Table 17. Amplification efficiency in control and mutation reactions: CRC cell lines

Sample		Intercept	Intercept standard error	Calculated slope	Standard error (slope)	Lower 2-sided 95% confidence limit (slope)	Upper 2-sided 95% confidence limit (slope)	Amplification efficiency	Difference in amplification efficiencies
12ALA	Control C _T	21.060	0.060	-1.008	0.007	-1.023	-0.993	0.989	0.03
	12ALA C _T	22.476	0.103	-0.987	0.013	-1.013	-0.961	1.019	
12ARG	Control C _T	20.825	0.083	-1.035	0.01	-1.056	-1.014	0.954	0.056
	12ARG C _T	23.237	0.083	-0.993	0.011	-1.016	-0.97	1.01	
12ASP	Control C _T	20.385	0.13	-1.013	0.16	-1.046	-0.98	0.982	-0.003
	12ASP C _T	21.347	0.065	-1.015	0.008	-1.032	-0.999	0.979	
12CYS	Control C _T	23.437	0.063	-0.981	0.01	-1.003	-0.96	1.026	0.032
	12CYS C _T	24.289	0.039	-0.961	0.006	-0.974	-0.947	1.058	
12SER	Control C _T	22.568	0.050	-1.003	0.008	-1.02	-0.986	0.996	0.105
	12SER C _T	25.212	0.087	-0.934	0.014	-0.963	-0.904	1.101	
12VAL	Control C _T	21.208	0.047	-0.995	0.006	-1.007	-0.983	1.007	0.033
	12VAL C _T	21.532	0.043	-0.972	0.005	-0.983	-0.961	1.04	
13ASP	Control C _T	23.207	0.056	-1.001	0.009	-1.02	-0.982	0.999	0.145
	12ASP C _T	26.466	0.106	-0.909	0.017	-0.945	-0.873	1.144	

Table 18. Amplification efficiency in control and mutation reactions: NSCLC samples

Sample		Intercept	Intercept standard error	Calculated slope	Standard error (slope)	Lower 2-sided 95% confidence limit (slope)	Upper 2-sided 95% confidence limit (slope)	Amplification efficiency	Difference in amplification efficiencies
12ALA	Control C _T	22.74	0.04	-0.15	0.02	-0.19	-0.11	0.94	0.069
	12ALA C _T	24.11	0.16	-1.06	0.07	-1.20	-0.93	1.01	
12ARG	Control C _T	21.92	0.03	-0.07	0.01	-0.09	-0.05	0.94	0.093
	12ARG C _T	24.44	0.02	-0.98	0.01	-0.96	-0.96	1.04	
12ASP	Control C _T	21.73	0.05	-0.13	-0.02	-0.17	-0.08	0.96	-0.001
	12ASP C _T	22.69	0.03	-0.97	0.01	-1.00	-0.95	0.96	
12CYS	Control C _T	21.73	0.04	-0.11	0.01	-0.14	-0.08	0.98	0.019
	12CYS C _T	22.77	0.03	-1.01	0.01	-1.03	-0.99	1.00	
12SER	Control C _T	22.03	0.05	-0.06	0.02	-0.10	-0.02	0.97	0.127
	12SER C _T	25.34	0.03	-0.97	0.01	-0.99	0.94	1.09	
12VAL	Control C _T	22.13	0.04	-0.03	0.02	-0.07	0.01	0.92	0.011
	12VAL C _T	23.34	0.08	-0.95	0.03	-1.01	-0.88	0.91	
13ASP	Control C _T	22.63	0.02	-0.02	0.01	0.001	-0.04	0.94	0.066
	12ASP C _T	25.14	0.07	-0.94	0.03	-1.00	-0.88	1.01	

Interfering substances

The objective of this study was to evaluate the impact of potentially interfering substances on the performance of the *therascreen* KRAS RGQ PCR Kit. This was performed by analyzing the impact of each substance on the ΔC_T values and mutation status of test samples by means of spiking experiments at various concentrations. Potential interfering substances from the DNA extraction process tested were Buffer AL, Buffer ATL, ethanol, paraffin wax, Proteinase K, Wash Buffer AW1, Wash Buffer AW2, and xylene. The final elution buffer from the kit, Buffer ATE, was also tested as the blank control.

At the concentrations expected to be encountered in normal use, none of the potentially interfering substances evaluated impacts on the ability of the *therascreen* KRAS RGQ PCR Kit to distinguish between mutation-positive and mutation-negative samples.

In addition to the interfering substances study, the potential effect of necrosis in clinical samples was assessed to determine if high levels of necrotic tissue in tumor samples impact the ability to generate valid data. From a total of 421 samples assessed as part of the Comparison to Analytical Reference Method studies, 29 samples had necrosis at a level >50% as determined by pathology review. Of these 29 samples, 28 returned valid results that were concordant to bi-directional Sanger sequencing. A single result was invalid due to insufficient DNA.

Cross-contamination

The objective of this study was to determine the extent of cross-contamination between DNA samples using the *therascreen* KRAS RGQ PCR Kit, which potentially leads to false-positive results. Potential sources of cross-contamination include the following:

- Sample extraction (e.g., scraping of slides)
- Pipetting of samples
- Closure (“capping”) of sample tubes
- Contamination of kit reagents during use
- Loading of assay tubes onto the Rotor-Gene Q MDx instrument

For this study, FFPE standards were used: the wild-type standard and the 12ALA standard (since the 12ALA reaction is the reaction with the lowest LOD in the kit).

The study consisted of 10 PCR runs designed to investigate the potential for contamination both within and between Rotor-Gene Q MDx instrument runs. In these test runs, tubes containing wild-type DNA were used to test for contamination from mutant DNA.

The results of this study indicated no detectable contamination in any of the wild-type DNA extracts intended to detect cross-contamination.

Exclusivity/cross-reactivity

The *therascreen* KRAS RGQ PCR Kit is comprised of 8 separate reactions, which includes a single control reaction that detects a non-polymorphic region of the KRAS gene, and 7 mutation-specific reactions. There is no reaction that specifically measures the wild-type KRAS sequence at codon 12 or 13. The KRAS “No Mutation Detected” result (i.e., wild-type) is determined from the absence of any of the 7 mutations resulting in a positive mutation result.

Therefore, it is necessary to demonstrate the amount of nonspecific amplification, or cross-reactivity that occurs in each reaction with excess amounts of KRAS wild-type DNA to ensure no false positive results occur. Similarly, nonspecific amplification is evaluated for KRAS mutations not intended to be detected by the assay. This demonstrates that the amount of cross-reactivity between mutant reactions does not result in erroneous mutation calls in the presence of excess amounts of mutant DNA. Since the DNA input for this assay is based on the control C_T range (21.92–32.00), the highest concentration of DNA input is based on having a control C_T value of approximately 22.

Nonspecific amplification/cross-reactivity: wild-type KRAS DNA

The amount of nonspecific amplification of wild-type DNA by reaction mixes designed to amplify specific mutations was addressed. A total of 60 replicates of wild-type FFPE cell line DNA and 60 NSCLC samples were evaluated at the highest concentration of amplifiable DNA input level using the *therascreen* KRAS RGQ PCR Kit.

The control C_T values were approximately 22–23. The results demonstrated that the ΔC_T values exceeded the established cutoffs and at least 95% of the wild-type replicates were correctly called.

Nonspecific amplification/cross-reactivity/exclusivity: mutation-positive KRAS DNA
 Mutant samples with a high concentration of input DNA were tested against all reaction mixes. DNA samples were prepared from each of the CRC and NSCLC FFPE cell lines so that the Control Reaction C_T corresponded to approximately 23. From these dilutions, 6 replicates of each mutation sample were evaluated. The percentage of mutation in the sample was governed by the percentage of mutant in the cell-line DNA.

The mean ΔC_T values presented in Table 19 and Table 20 demonstrate that there is cross-reactivity between mutant reactions. In all cases, the results show that the correct mutation was called with the matched mutation reaction (i.e., the smallest ΔC_T value was the correct mutation call). All other test cases were either not detected or outside the ΔC_T threshold.

Table 19. Cross-reactivity (ΔC_T) between mutation reactions using CRC FFPE cell line DNA at the high input range

Mutant DNA	Cutoff	Assay ΔC_T						
		12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
12ALA	8	1.42*	12.66	NA	5.81[†]	2.78[†]	6.31[†]	13.21
12ASP	6.6	12.56	2.42*	NA	NA	13.44	11.21	13.55
12ARG	8	13.12	11.56	1.12*	11.42	NA	13.43	12.66
12CYS	8	14.2	12.48	9.23	0.98*	NA	7.96[†]	12.88
12SER	8	NA	13.39	13.31	NA	3.02*	12.99	13.97
12VAL	7.5	6.83[†]	NA	NA	NA	13.38	0.28*	13.74
13ASP	7.5	NA	13.29	13.89	NA	NA	14.36	4.5*

NA: No cross-reaction.

* ΔC_T values from matched reactions.

[†] ΔC_T from cross-reactive reactions below the cutoff.

Table 20. Cross-reactivity (ΔC_T) between mutation reactions using NSCLC FFPE cell line DNA at the high input range

Mutant DNA	Cutoff	Assay ΔC_T						
		12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
12ALA	8	1.31*	12.8	NA	5.01†	2.26†	5.57†	12.65
12ASP	6.6	12.61	1.66*	NA	NA	NA	10.3	12.60
12ARG	8	12.98	11.08	0.81*	11.24	NA	12.66	12.62
12CYS	8	NA	12.22	7.84†	0.56*	NA	13.06	11.84
12SER	8	NA	12.87	13.21	NA	1.93*	13.25	12.93
12VAL	7.5	5.93†	14.29	NA	NA	13.14	0.45*	12.39
13ASP	7.5	NA	NA	NA	NA	NA	NA	2.02*

NA: No cross-reaction.

* ΔC_T values from matched reactions.

† ΔC_T from cross-reactive reactions below the cutoff.

Repeatability and reproducibility

The objectives of this study were to demonstrate the precision of the *therascreen* KRAS RGQ PCR Kit within-laboratory (repeatability) and between-laboratories (reproducibility). Both correctness of mutation call results and the precision of ΔC_T values (the difference in C_T values between a Mutation Reaction and the Control Reaction) are reported.

CRC

Clinical CRC samples were used for this evaluation. One wild-type and one sample for each mutation were tested with the *therascreen* KRAS RGQ PCR Kit using 2 operators at each of 3 sites, testing all samples and controls on 3 lots of *therascreen* KRAS RGQ PCR Kits, each day for 5 days, with 2 runs per day and with 2 replicates of each sample on each run. The C_T and ΔC_T values obtained for each reaction in each sample were also analyzed by variance component analysis.

Reproducibility of the *therascreen* KRAS RGQ PCR Kit was demonstrated for low level mutant (3x LOD) and wild-type samples with at least 39/40 correct mutation calls for all assays across multiple lots, platforms, and operators, for both within and between laboratory. The estimated proportion of 3x LOD samples testing as mutant and wild-type samples was reported overall and within each of the sites. For all assays and sample combinations, at least 79 out of 80 replicates gave the correct mutation call (Table 21).

Table 21. Correct calls overall

Sample	Mutation assay correct calls						
	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP
Mutant 3x LOD	79/80	80/80	80/80	79/80	80/80	80/80	80/80
Wild-type (low)	80/80	79/80	80/80	80/80	79/80	79/80	80/80

NSCLC

For each of the 7 KRAS NSCLC mutations, 3 samples representing each of the 3 sample acquisition method types (resection, CNB and FNA) were used. An additional 6 wild-type clinical samples (2 samples representing each of the 3 sample acquisition method types) were used to create wild-type DNA diluent pools.

Multiple extracts were pooled for each of the mutation samples to create a single sample pool per mutation. Each mutation sample pool was diluted to generate test samples at mutation levels of 1x LOD and 3x LOD.

Laboratories used in this study were at 3 different sites. Laboratory conditions were varied at each site by the use of 2 Rotor-Gene Q MDx instruments, 2 operators, 2 lots of the *therascreen* KRAS RGQ PCR Kit, and 2 runs per day (per operator) over 16 non-consecutive days.

For all assays and sample combinations, at least 284 out of 288 replicates gave the correct mutation call. The overall proportion of correct calls, all assays combined, for the 1x LOD group was 100%. The overall proportion of correct calls, all assays combined, for the 3x LOD group was 99.6%. The overall proportion of correct calls for the no-mutation detected (wild-type) samples was 100% (Table 22).

Table 22. Correct calls for 1x LOD, 3x LOD, and wild-type

Mutation level	Assay	Correct calls	Correct calls, %	Lower two-sided 90% CI
1x LOD	12ALA	288/288	100	98.97
	12ARG	288/288	100	98.97
	12ASP	288/288	100	98.97
	12CYS	284/284	100	96.85
	12SER	284/284	100	96.85
	12VAL	288/288	100	98.97
	13ASP	288/288	100	98.97
3x LOD	12ALA	288/288	100	98.97
	12ARG	288/288	100	98.97
	12ASP	288/288	100	98.97
	12CYS	284/288	98.61	96.85
	12SER	284/288	98.61	96.85
	12VAL	288/288	100	98.97
	13ASP	287/287	100	98.96
Wild-type		285/285	100	98.95

Sample handling variability

The objective of this study was to assess the effect of sample handling variability, specifically DNA extraction, on the *therascreen* KRAS RGQ PCR Kit. This study complements the repeatability and reproducibility study by analyzing the sample handling variability when the

same clinical FFPE sections and FFPE cell line sections were processed at 3 sites followed by testing with the *therascreen* KRAS RGQ PCR Kit.

CRC

Thirty sequential 5 μ m sections were cut from each of 10 FFPE CRC samples (3 wild-type and 1 per mutation). Sections were randomized to 1 of 3 testing sites so that each site received 10 sections per FFPE sample (100 sections total). Of the 300 DNA extractions tested, 298 samples were valid. There was 99.33% concordance with respect to the KRAS mutation calls between the 3 sites.

A comparison by site of mean ΔC_T values for mutant and wild-type samples showed very close agreement for the results. The results demonstrate the agreement of the DNA extraction procedure and sample processing in conjunction with the *therascreen* KRAS RGQ PCR Kit.

NSCLC

There were 13 clinical NSCLC samples (3 x 12ASP, 3 x 12CYS, 4 x 12VAL and 3 wild-type) and 4 FFPE cell-line samples (12ALA, 12ARG, 12SER and 13ASP) used in this study. Samples represented the different methods of acquisition: surgical resection, FNA and CNB. Cell lines were used to represent rare mutations where clinical NSCLC tissue was not available.

Three batches of 20 FFPE sections were then randomly distributed to the 3 sites. At each of the 3 sites, DNA extraction was performed on a batch of 20 FFPE sections (10 pairs) per mutation and wild-type.

When all sample preparations at 3 individual test sites were tested with the *therascreen* KRAS RGQ PCR Kit, each of the 7 mutations and wild-type samples was identified with the correct mutation call. The overall call for each of the 7 mutations and wild-type samples was 100%, demonstrating inter-site consistency for DNA extraction and mutation detection using the *therascreen* KRAS RGQ PCR Kit.

Equivalence of sample acquisition methods (NSCLC only)

The purpose of this study was to assess whether the mutation call for NSCLC samples determined by the *therascreen* KRAS RGQ PCR Kit was affected by the sample acquisition method. The 3 sample acquisition methods assessed in this study were resection, FNA and CNB.

For this study, “patient-matched” CNB and FNA samples were derived from surgically resected tumor samples to enable the same tumor to be collected by the 3 acquisition methods. A total of 169 resection, 169 CNB, and 169 FNA samples were available for this study.

Each sample was extracted and tested with the KRAS Control assay. Each sample giving a valid result (169 resections, 169 CNB, and 164 FNA) was tested with all 8 KRAS assays.

In addition, for each of the clinical FFPE NSCLC samples, the extracted DNA used for the *therascreen* KRAS RGQ PCR Kit analysis was also assessed by bi-directional Sanger sequencing to determine the level of concordance between the *therascreen* KRAS RGQ PCR Kit and bi-directional Sanger sequencing. Across all sample types, the *therascreen* KRAS RGQ PCR Kit accurately determined the mutation status versus bi-directional Sanger sequencing with an overall percentage agreement rate of 96.96%.

The results from this study demonstrate that the *therascreen* KRAS RGQ PCR Kit provides equivalent results across the 3 collection methods studied as indicated by the pair-wise percent overall agreement rates:

- CNB vs. FNA 97.52 (confidence limits 94.41–99.15)
- CNB vs. resection 96.39 (confidence limits 92.99–98.41)
- FNA vs. resection 98.76 (confidence limits 96.14–99.78)

References

Cited references

1. Hilger, R.A., et al. (2002) The Ras-Raf-MEK-ERK pathway in the treatment of cancer. *Onkologie* **25**, 511.
2. Bachireddy, P., et al. (2005) Getting at MYC through RAS. *Clin. Cancer Res.* **11**, 4278.
3. Han, S.-W. et al. (2006) Optimization of patient selection for gefitinib in non-small cell lung cancer by combined analysis of epidermal growth factor receptor mutation, K-ras mutation, and AKT phosphorylation. *Clin. Cancer Res.* **12**, 2538.
4. Pao, W. et al. (2005) KRAS mutations and primary resistance of lung adenocarcinomas to gefitinib or erlotinib. *PLoS Medicine* **2**, 57.
5. Newton, C.R. et al. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* **17**, 2503.
6. Whitcombe, D. et al. (1999) Detection of PCR products using self-probing amplicons and fluorescence. *Nature Biotech.* **17**, 804.
7. Catalog of Somatic Mutations in Cancer: www.sanger.ac.uk/genetics/CGP/cosmic.
8. Clinical and Laboratory Standards Institute (CLSI) (2004). *Protocols for Determination of Limits of Detection and Limits of Quantitation: Approved Guideline. CLSI Document EP17-A*. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

Useful references

Amado, R.G. (2008) Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J. Clin. Oncol.* **26**, 1626.

Benvenuti, S. et al. (2007) Oncogenic activation of the RAS/RAF signaling pathway impairs the response of metastatic colorectal cancers to anti-epidermal growth factor receptor antibody therapies. *Cancer Res.* **67**, 2643.

Bokemeyer, C. et al., (2008) K-RAS status and efficacy of first-line treatment of patients with metastatic colorectal cancer (mCRC) with FOLFOX with or without cetuximab: The OPUS experience. *J. Clin. Oncol.* **26** (May 20 suppl; abstr 4000).

Chaft, J.E. et al. (2013) Phase II trial of neoadjuvant bevacizumab plus chemotherapy and adjuvant bevacizumab in patients with resectable nonsquamous non-small-cell lung cancers. *J. Thorac. Oncol.* **8**, 1084.

Clinical and Laboratory Standards Institute (CLSI) (2008). *User Protocol for Evaluation of Qualitative Test Performance: Approved Guideline*, 2nd ed. CLSI Document EP12-A2. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

Clinical and Laboratory Standards Institute (CLSI) (2004). *Evaluation of Precision Performance of Quantitative Measurement Methods: Approved Guideline*, 2nd ed. CLSI Document EP05-A2. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

De Roock, W. et al. (2007) KRAS mutations preclude tumor shrinkage of colorectal cancers treated with cetuximab. *J. Clin. Oncol.* **25**, 4132.

De Roock, W. et al. (2008) KRAS wild-type state predicts survival and is associated to early radiological response in metastatic colorectal cancer treated with cetuximab. *Ann. Oncol.* **19**, 508.

Di Fiore, F. et al. (2007) Clinical relevance of KRAS mutation detection in metastatic colorectal cancer treated by cetuximab plus chemotherapy. *Br. J. Cancer* **96**, 1166.

Dingemans, A.M. et al. (2013) A phase II study of sorafenib in patients with platinum-pretreated, advanced (Stage IIIb or IV) non-small cell lung cancer with a KRAS mutation. *Clin. Cancer Res.* **3**, 743.

Finocchiaro, G. et al. (2007) EGFR, HER2, and Kras as predictive factors for cetuximab sensitivity in colorectal cancer. *J. Clin. Oncol.* **25**, 4021.

Jänne, P.A. et al. (2013) Selumetinib plus docetaxel for KRAS-mutant advanced non-small-cell lung cancer: a randomised, multicentre, placebo-controlled, phase 2 study. *Lancet Oncol.* **1**, 38.

Karapetis C. et al. (2008) KRAS mutation status is a predictive biomarker for cetuximab benefit in the treatment of advanced colorectal cancer. Results from NCIC CTG CO.17: A phase III trial of cetuximab versus best supportive care. 10th World Congress on Gastrointestinal Cancer: Abstract o-037. Presented June 27, 2008.

Khambata-Ford, S. et al. (2007) Expression of Epiregulin and Amphiregulin and K-ras mutation status predict disease control in metastatic colorectal cancer patients treated with cetuximab. *J. Clin. Oncol.* **25**, 3230.

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- Lièvre A. et al. (2008) KRAS mutations as an independent prognostic factor in patients with advanced colorectal cancer treated with cetuximab. *J. Clin. Oncol.* **26**, 374.
- Lievre, A. et al. (2006) KRAS mutation status is predictive of response to cetuximab therapy in colorectal cancer. *Cancer Res.* **66**, 3992.
- Reckamp, K.L. et al. (2014) A phase 2 trial of dacomitinib (PF-00299804), an oral, irreversible pan-HER (human epidermal growth factor receptor) inhibitor, in patients with advanced non-small cell lung cancer after failure of prior chemotherapy and erlotinib. *Cancer.* **120**, 1145.
- Tejpar, S. et al. (2008) Relationship of efficacy with K-RAS status (wild type versus mutant) in patients with irinotecan-refractory metastatic colorectal cancer (mCRC), treated with irinotecan (q2w) and escalating doses of cetuximab (q1w): The EVEREST experience (preliminary data). *J. Clin. Oncol.* **26**, (May 20 suppl; abstr 4001).
- Thelwell, N. et al. (2000) Mode of action and application of Scorpion primers to mutation detection. *Nucleic Acids Res.* **28**, 3752.
- Van Cutsem, E. et al. (2008) K-RAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: The CRYSTAL experience. *J Clin Oncol.* **26**, (May 20 suppl; abstr 2).

Symbols

The following symbols may appear on the packaging and labeling:



<N>

Contains reagents sufficient for <N> reactions



Use by



In vitro diagnostic medical device



Catalog number



Lot number



Material number



Contains



Number



Authorized Representative

Rn

R is for revision of the Handbook and n is the revision number



Temperature limitation



Manufacturer



Consult instructions for use



Caution

Contact Information

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

Appendix 1: *therascreen* KRAS RGQ PCR Kit Manual Protocol

This section contains instructions for using the *therascreen* KRAS RQG PCR Kit with RGQ software version 2.3 in the open mode (i.e., without using the KRAS Assay Package).

General information

- For materials required, see “Materials Required but Not Provided.”
- For full instructions on sample preparation and sample layout, see sections “Protocol: DNA sample assessment” and “Protocol: Detection of KRAS mutations.”

Protocol: Creating a temperature profile

Before starting, create a temperature profile for the KRAS analysis. The cycling parameters are the same for both Sample Assessment and Mutation Assessment.

Procedure

The cycling parameters are shown in Table 23.

Table 23. Cycling parameters

Cycles	Temperature	Time	Data acquisition
1	95°C	15 minutes	None
40	95°C	30 seconds	None
	60°C	60 seconds	Green and yellow

1. **Double-click the Rotor-Gene Q Series Software 2.3 software icon on the desktop of the laptop connected to the Rotor-Gene Q MDx instrument. Select the “Advanced” tab in the “New Run” dialog box that appears.**

2. To create a new template, select “Empty Run” and then click “New” to enter the “New Run Wizard.”
3. Select 72-Well Rotor as the rotor type. Confirm that the locking ring is attached and check the “Locking Ring Attached” box. Click “Next” (Figure 21).

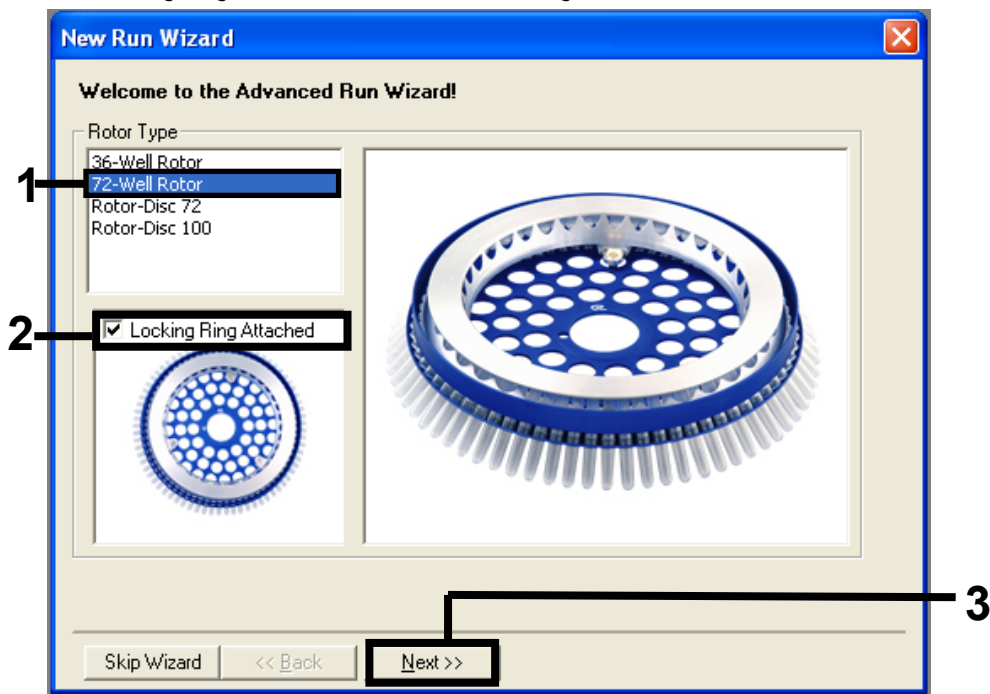


Figure 21. The “New Run Wizard” dialog box. 1 = “Rotor type, 2 = “Locking Ring Attached box,” 3 = “Next.”

4. Enter the name of the operator. Add any notes and enter the reaction volume as 25. Ensure that “Sample Layout” reads “1, 2, 3...”. Click “Next” (Figure 22).

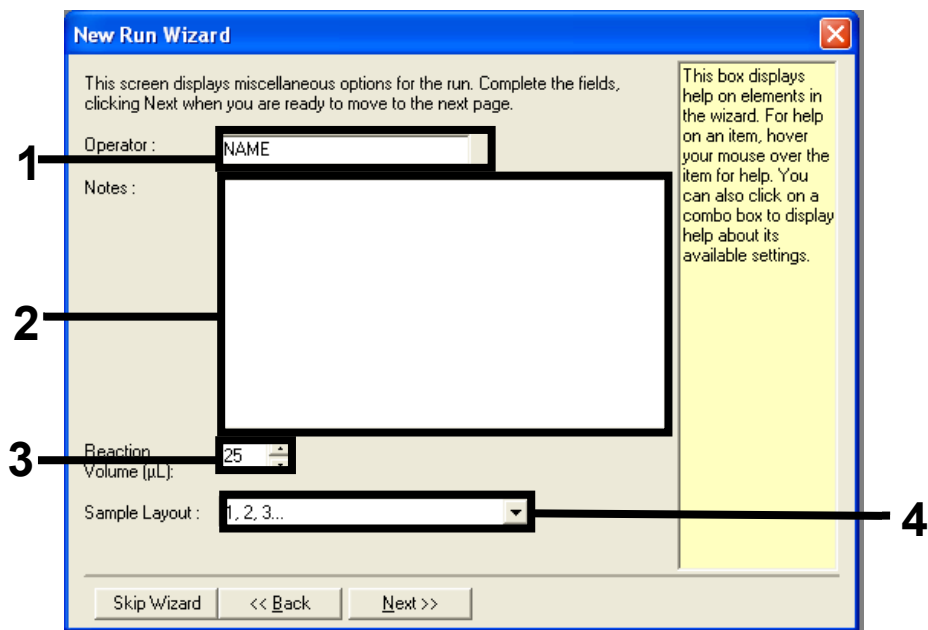


Figure 22. Entering operator name and reaction volumes. 1="Operator" dialog field, 2 = "Notes" dialog field, 3 = "Reaction Volume" field, 4 = "Sample Layout," 5 = "Next."

5. Click "Edit Profile" in the "New Run Wizard" dialog box (Figure 23) and program the temperature profile according to the information in the following steps.

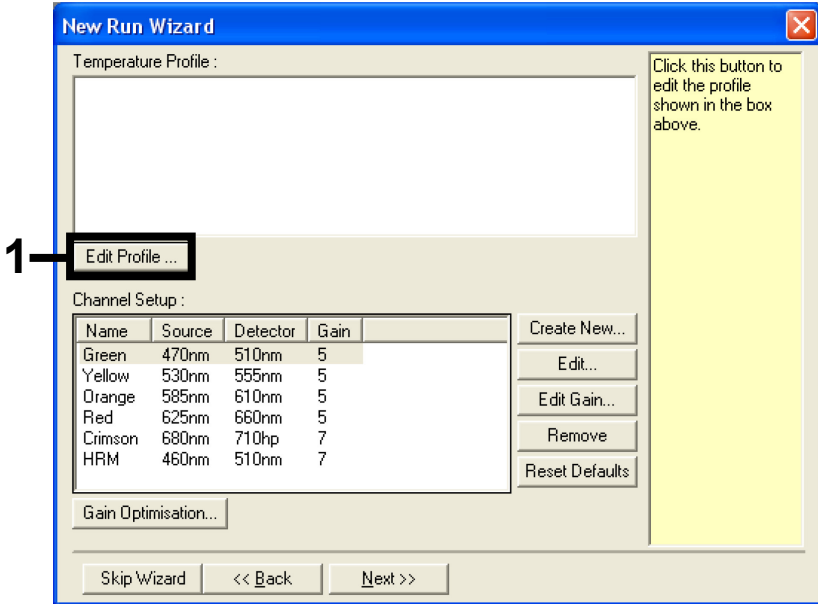


Figure 23. Editing the profile.

6. Click "Insert after" and select "New Hold at Temperature" (Figure 24).

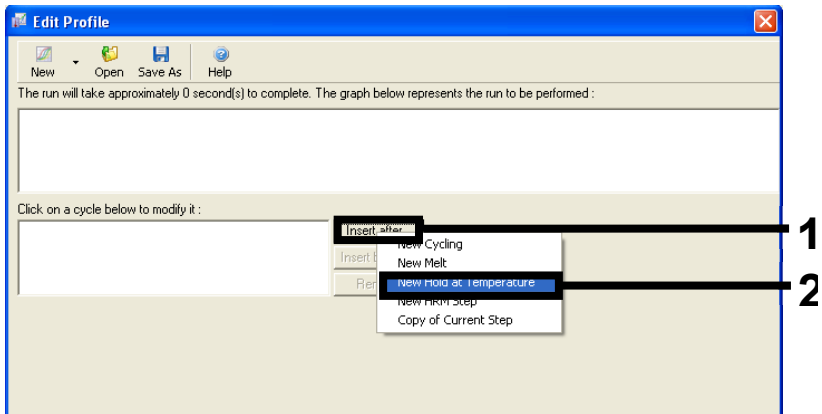


Figure 24. Inserting an initial incubation step. 1 = "Insert after," 2 = "New Hold at Temperature."

7. Change “Hold Temperature” to 95°C and “Hold Time” to 15 mins 0 secs. Click “Insert After” and then select “New Cycling” (Figure 25).

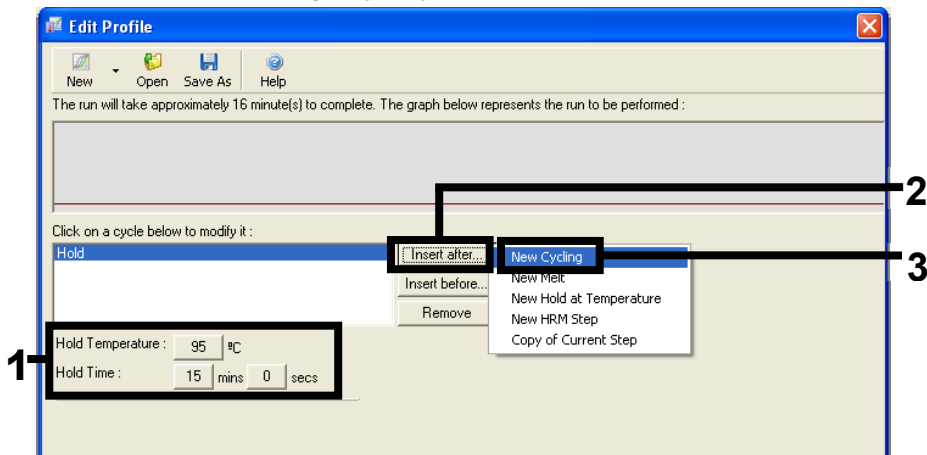


Figure 25. Initial incubation step at 95°C. 1 = “Hold Temperature and Hold Time,” 2 = “Insert after,” 3 = “New Cycling.”

8. Change the number of cycle repeats to 40. Select the first step and set to “95°C for 30 secs” (Figure 26).

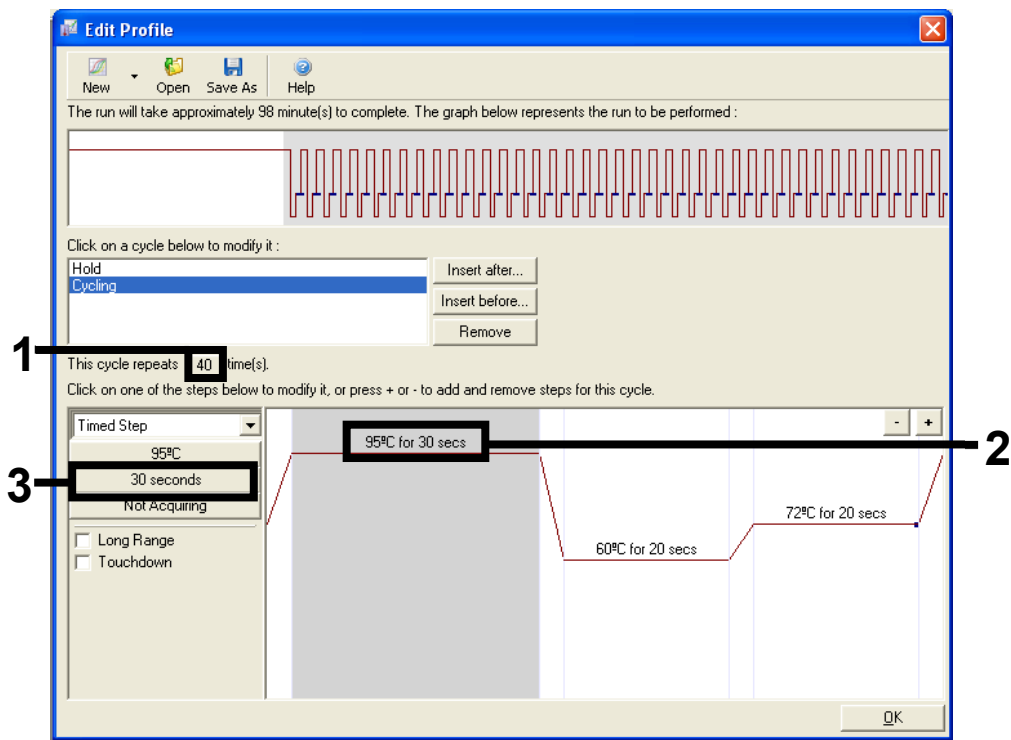


Figure 26. Cycling step at 95°C. 1 = "Cycle repeats" box, 2 = "step one temperature setting, 3 = step one time setting.

9. Highlight the second step and set to "60°C for 60 secs." Enable data acquisition during this step by selecting "Not Acquiring" (Figure 27).

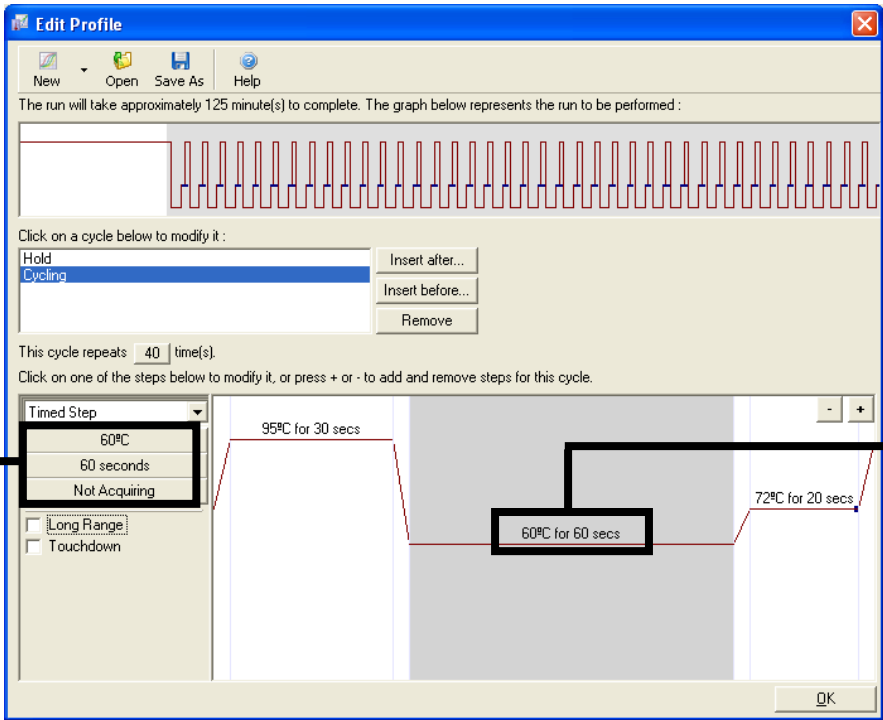


Figure 27. Cycling step at 60°C. 1 = second step temperature and time setting, 2 = Not Acquiring.

10. Set "Green" and "Yellow" as acquiring channels by selecting ">" to transfer them from the "Available Channels" list. Click "OK" (Figure 28).

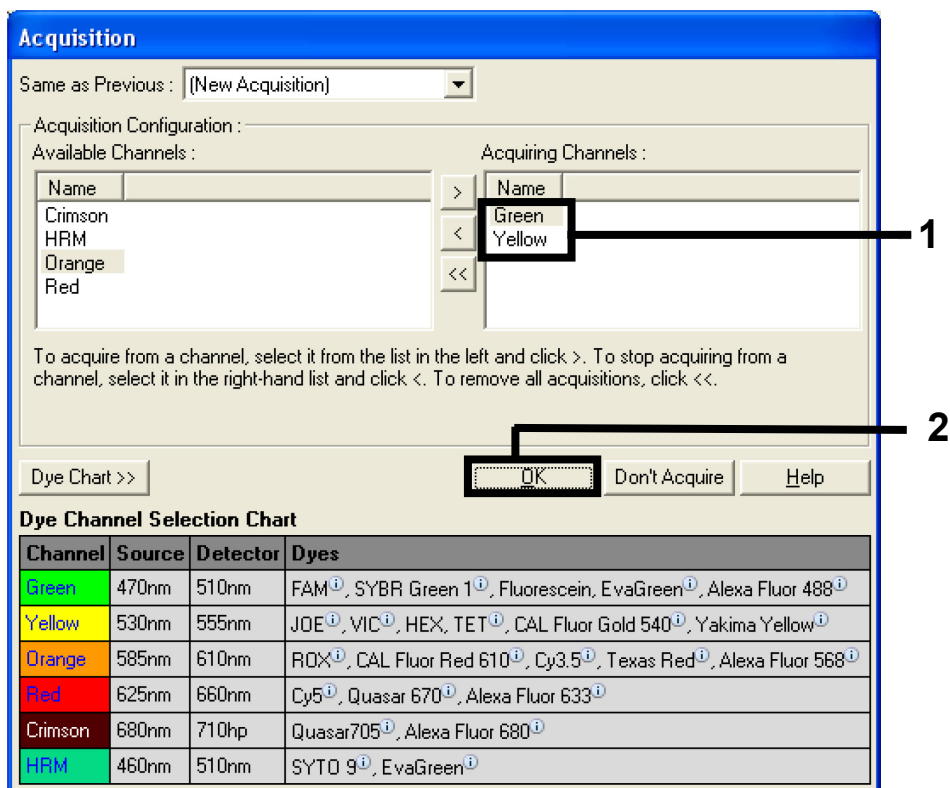


Figure 28. Acquiring at cycling step of 60°C.

11. Highlight the third step and delete by clicking “-.” Click “OK” (Figure 29).

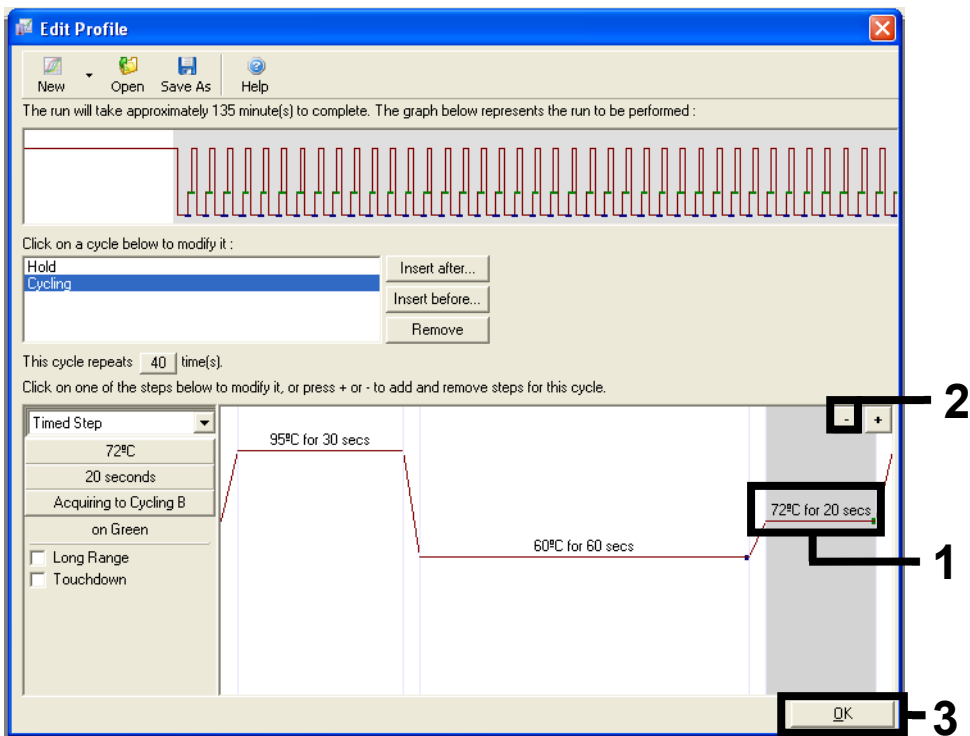


Figure 29. Removal of extension step.

12. In the next dialog box, click "Gain Optimization" (Figure 30).

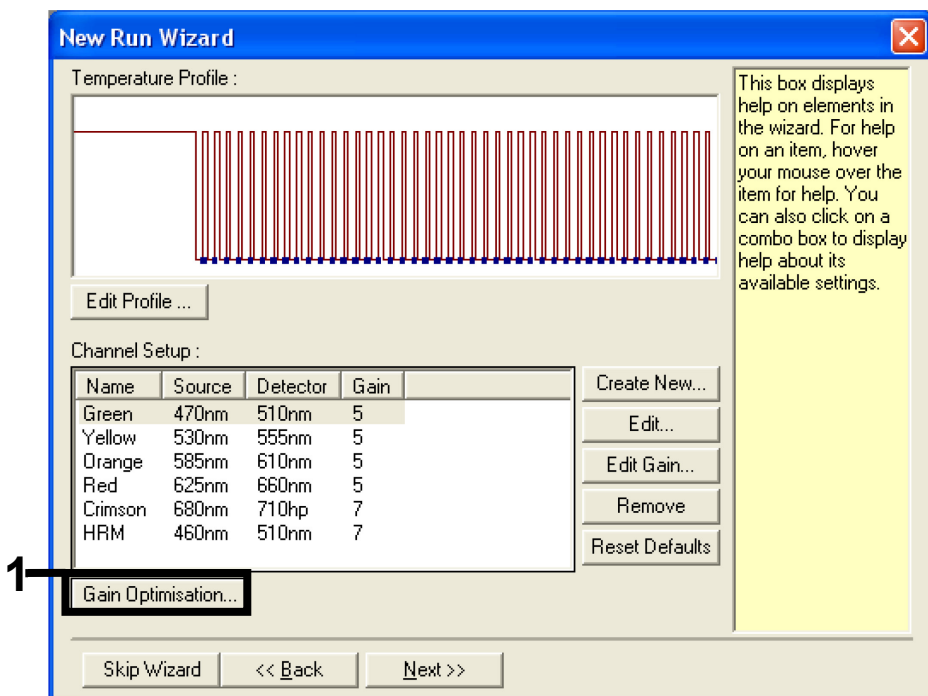


Figure 30. "Gain Optimization."

13. Click "Optimize Acquiring." Channel settings are displayed for each channel. Accept these default values by clicking "OK" for both channels (Figure 31).

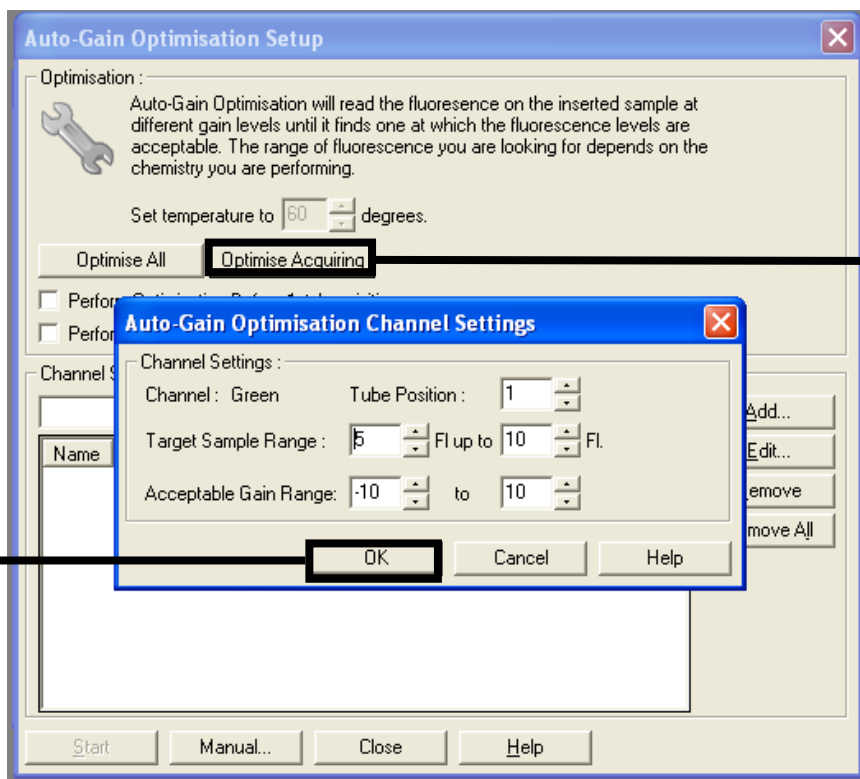


Figure 31. "Auto-gain Optimization" for the green channel.

14. Check the "Perform Optimization before 1st Acquisition" box and then click "Close" to return to the wizard (Figure 32).

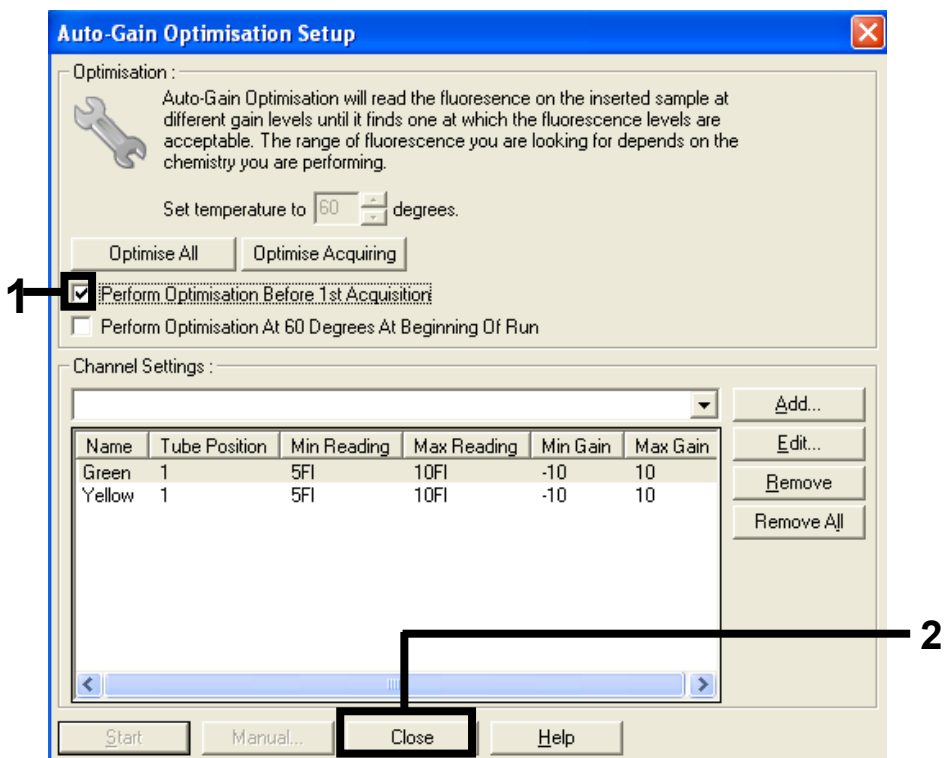


Figure 32. Selection of green and yellow channels.

15. Click “Next” to save the template in an appropriate location by selecting “Save Template.”

Protocol: Sample assessment (manual)

This protocol is used to assess the total amplifiable DNA in samples and should be performed prior to KRAS mutation analysis.

- Prepare samples as described in “Protocol: DNA sample assessment.”

- Set up the PCR run on the Rotor-Gene Q MDx instrument as described in “Protocol: *therascreen* KRAS PCR RGQ setup.”
- After the run is complete, analyze the data according to the instructions in “Sample assessment data analysis.”

Protocol: KRAS mutation detection (manual)

Once a sample has passed the sample assessment, it can be tested to detect KRAS mutations.

- Prepare samples as described in “Protocol: Detection of KRAS mutations.”
- Set up the PCR run on the Rotor-Gene Q MDx as described in “Protocol: *therascreen* KRAS PCR RGQ setup.”
- After the run is complete, analyze the data according to the instructions in section “KRAS mutation detection analysis.”

Protocol: *therascreen* KRAS PCR RGQ setup

1. **Open the Rotor-Gene Q series software 2.3 and the appropriate created temperature profile.**

Create the temperature profile according to “Protocol: Creating a temperature profile.”

2. **Ensure the correct rotor is selected, and check the box to confirm the locking ring is attached. Click “Next” (Figure 33).**

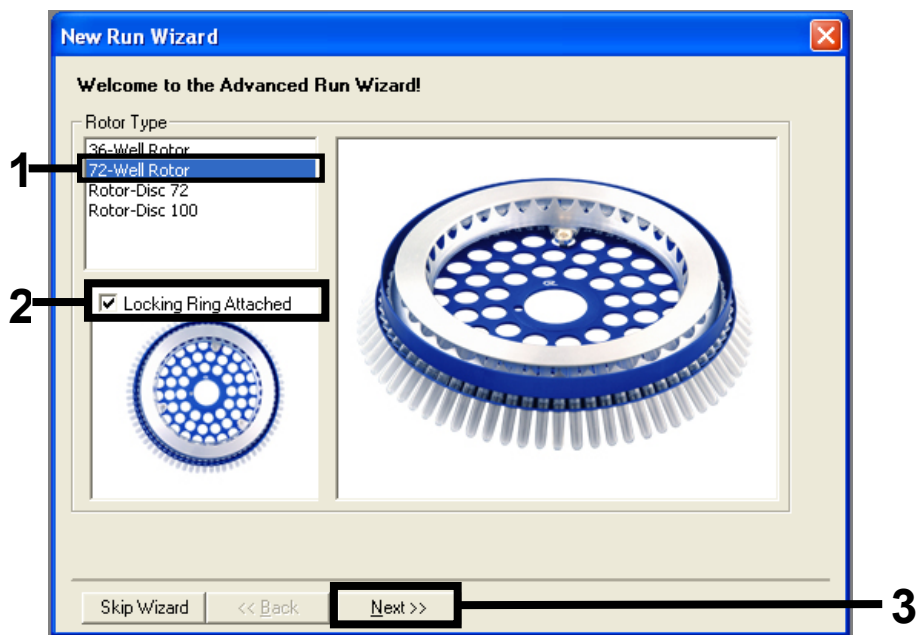


Figure 33. The “New Run Wizard” dialog box and welcome screen. 1 = Rotor type, 2 = “Locking Ring Attached box,” 3 = “Next.”

3. Enter the name of the operator. Add any notes and check that the reaction volume is set to 25 and the “Sample Layout” box reads “1, 2, 3...”. Click “Next” (Figure 34).

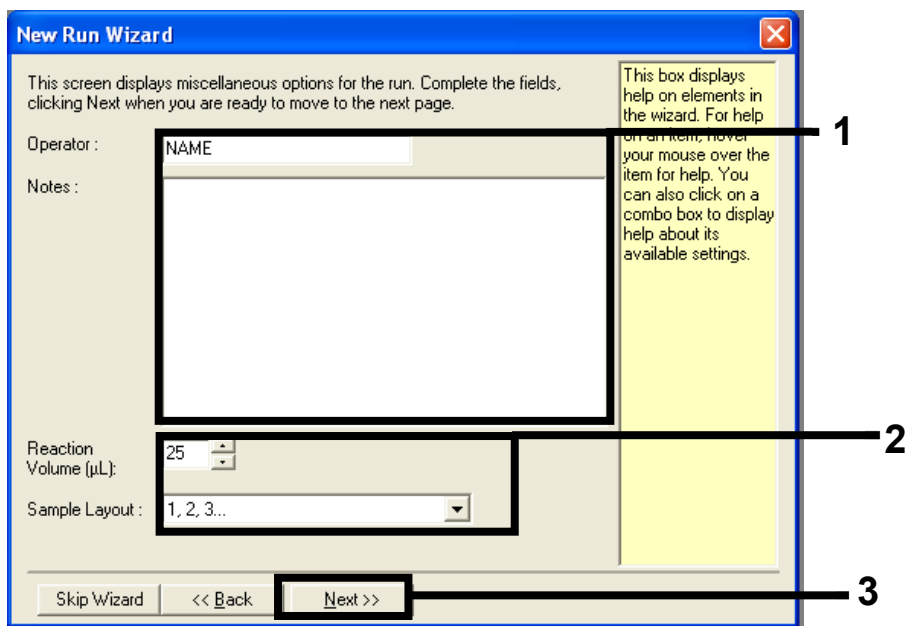


Figure 34. The “New Run Wizard” dialog box. 1 = “Operator” and “Notes” fields, 2 = “Reaction Volume” and “Sample Layout” fields, 3 = “Next.”

- 4. This next window allows editing of the temperature profile. No editing is required as the temperature profile has been created according to the instructions in “Protocol: Creating a temperature profile.” Click “Next” (Figure 35).**

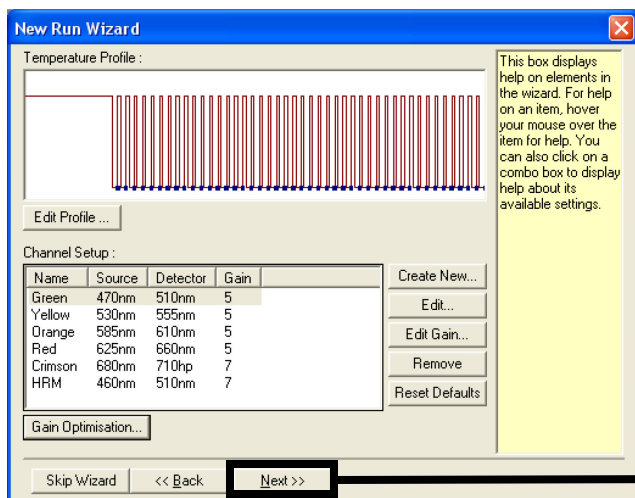


Figure 35. The “New Run Wizard” dialog box and temperature editing screen. 1 = “Next.”

5. Check the summary and click “Start Run” to save the run file and start the run (Figure 36).

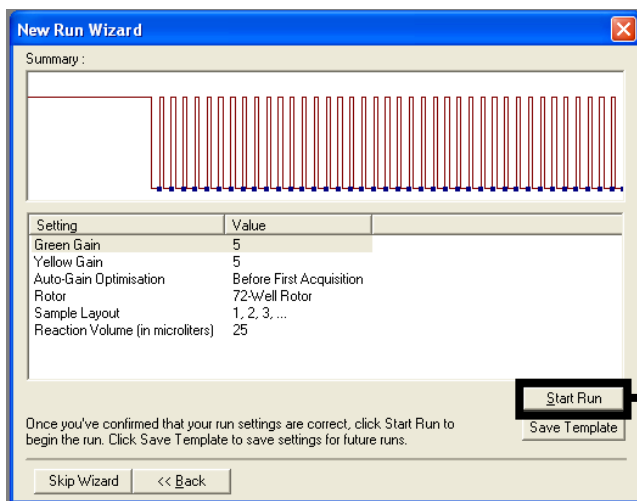


Figure 36. The “New Run Wizard” dialog box. 1 = “Start Run.”

6. **After the run starts, a new window appears in which you can either enter sample names now or click “Finish,” and enter them later by selecting the “Sample” button during the run or once the run is complete.**

Clicking “Finish and Lock Samples” will prevent you from editing the sample names. The user should take particular care when entering sample names to ensure correct sample testing and analysis.

Note: When naming samples, empty wells should be left blank in the “Name” column.

7. **After the run is complete, analyze data according to sections “Sample assessment data analysis,” or “KRAS mutation detection analysis,” as appropriate.**
8. **If quantification reports are required, click the “Reports” icon of the toolbar in the Rotor-Gene Q run file.**

Interpretation of Results (Manual)

After the sample assessment run or the mutation analysis run is complete, analyze the data according to the following procedure.

Software analysis settings

1. **Open the appropriate file using the Rotor-Gene Q series software 2.3.**
2. **If you haven’t already named your samples prior to performing the run, click “Edit Samples.”**
3. **Insert the names of your samples in the “Name” column.**
4. **Click “Analysis.” On the analysis page, click “Cycling A. Yellow” to view the HEX channel.**
5. **Click “Named On.”**
Note: This ensures that empty wells do not feature in the analysis.
6. **Select “Dynamic Tube.”**
7. **Select “Linear Scale.”**
8. **Click “Outlier Removal” and enter “10%” for the “NTC Threshold.”**

9. Set the threshold at 0.05 and check the HEX C_T values.
10. On the analysis page, click “Cycling A. Green” to view the FAM channel.
11. Check that “Dynamic Tube” is highlighted. Click “Linear Scale.”
12. Click “Outlier Removal” and enter “10%” for the “NTC Threshold.”
13. Set the threshold at 0.05 and check the FAM C_T values.

Sample assessment data analysis

Run control analysis

Refer to the “Run control analysis” flowchart in Figure 37.

- **Negative control:** To ensure there is no contamination of the reaction mix, the No Template Control must not generate a C_T value in the green channel below 40. To ensure that the plate was set up correctly, the NTC must show amplification of 31.91–35.16 in the yellow channel. The values specified are within and including those values.
- **Positive control:** The KRAS Positive Control (PC) must give a C_T value of 23.5–29.5 in the green channel in each of the 8 assays. The values specified are within and including those values. A value outside this range indicates an assay setup problem and is therefore a run failure.

Note: Sample data must not be used if either of these two run controls has failed.

Provided that both run controls are valid, each sample C_T value must be within the range of 21.92–32.00 in the green channel. If the sample is out of this range, the following guidance is provided.

Sample analysis – control assay

- Sample control assay C_T of <21.92: Samples with a control C_T of <21.92 must be diluted as this represents the lower end of the validated assay range. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range on the basis that diluting by half will increase the C_T by 1. If the sample is close to 21.92, dilution is recommended in order to ensure a result is obtained from the

sample test (KRAS mutation detection) run. Samples should be diluted using the water provided in the kit (Nuclease-Free Water for Dilution [Dil.]).

- Sample control assay C_T of >32 : Re-extraction of the sample is recommended as insufficient starting DNA template will be present to detect all mutations at the stated cutoff values for the assay.

KRAS mutation detection analysis

Run control analysis

Refer to the “Run control analysis” flowchart (Figure 37).

- **Negative control:** To ensure there is no contamination of the reaction mix, the No Template Control must not generate a C_T value in the green channel below 40. To ensure that the plate was set up correctly, the NTC must show amplification of 31.91–35.16 in the yellow channel. The values specified are within and including those values.
- **Positive control:** The KRAS Positive Control (PC) must give a C_T value of 23.5–29.5 in the green channel in each of the 8 assays. The values specified are within and including those values. A value outside this range indicates an assay setup problem and is therefore a run failure.

Note: Sample data must not be used if either of these 2 run controls has failed.

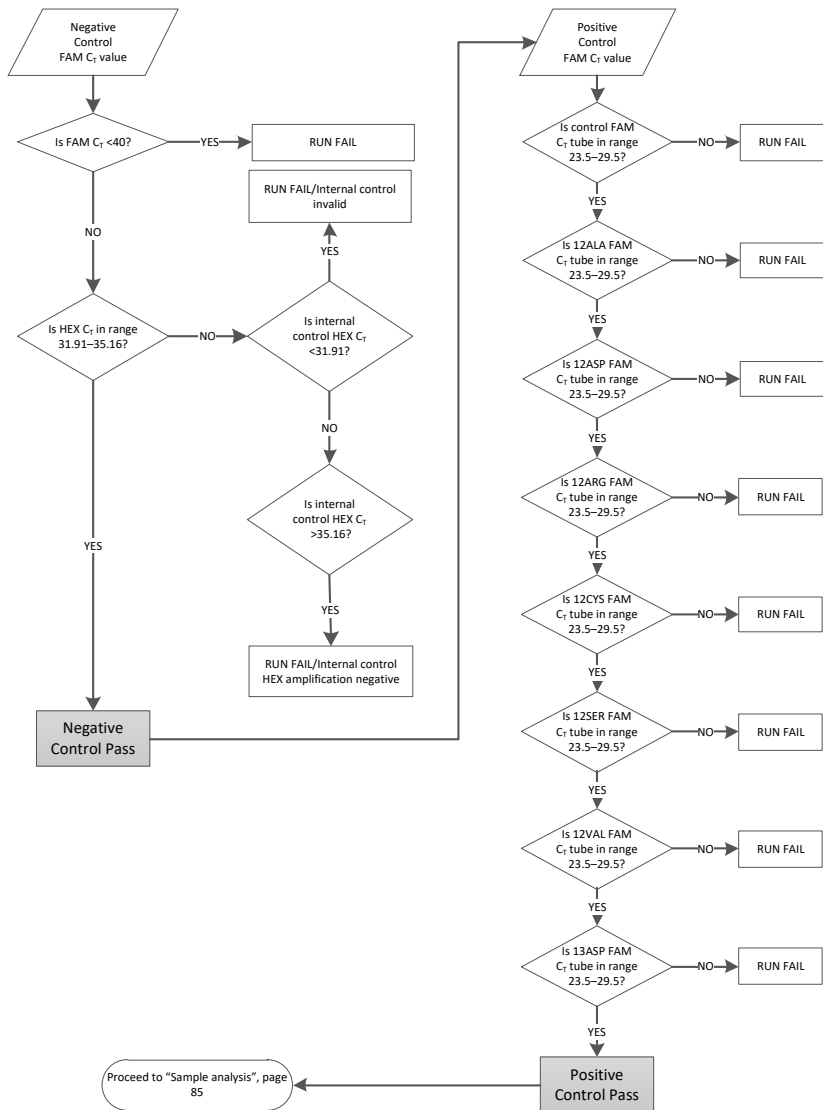


Figure 37. Run control analysis flowchart.

Sample analysis

Refer to the “Sample analysis” flowchart in Figure 38.

Sample control FAM C_T value

Provided that both run controls are valid for the control assay, each sample control C_T value must be within the range of 21.92–32.00 in the green channel.

If the sample is out of this range, the following guidance is provided.

- **Sample control assay C_T <21.92:** Samples with a control C_T of <21.92 will overload the mutation assays and must be diluted. To detect each mutation at a low level, over-concentrated samples must be diluted to fall within the above range 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 (Nuclease-Free Water for Dilution [Dil.]).
- **Sample control assay C_T >32:** Interpret with caution as very low-level mutations may not be detected.

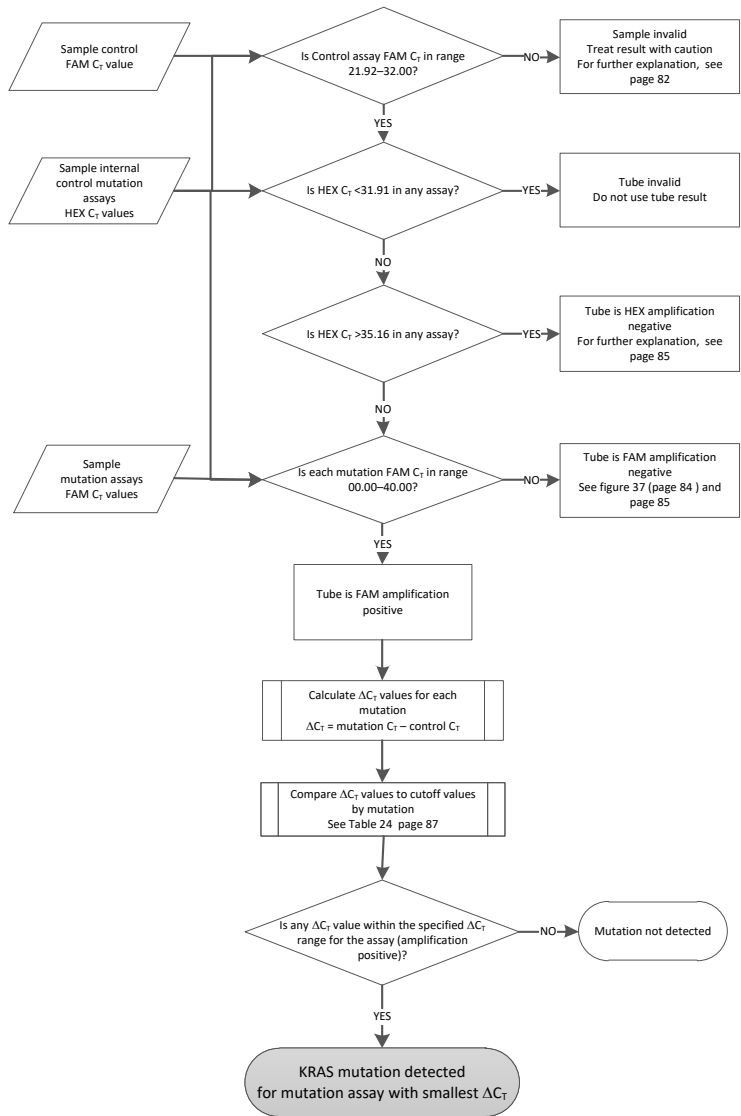


Figure 38. Sample analysis flowchart.

Sample internal control mutation assays HEX C_T value

Refer to the “Sample analysis” flowchart in Figure 38.

All wells of each sample must be analyzed. Check that each well generates a HEX signal from the internal control. There are 3 possible outcomes.

- If the internal control C_T falls within the specified range (31.91–35.16), it is HEX amplification positive.
- If the internal control C_T is above the specified range (>35.16), it is HEX amplification negative.
- If the internal control C_T is below the specified range (<31.91), it is invalid.

If the internal control failure is due to PCR inhibition, diluting the sample may reduce the effect of inhibitors, but it should be noted that this would also dilute the target DNA. A tube of Water for Sample Dilution (Dil.) is included with the kit.

Sample mutation assays FAM C_T value

The FAM values for all 7 reaction mixes should be checked against the values listed in Table 24.

Table 24. Acceptable sample mutation reaction values (FAM)*

Assay	Acceptable C _T range	ΔC _T range
12ALA	0.00–40.00	≤8.00
12ASP	0.00–40.00	≤6.60
12ARG	0.00–40.00	≤8.00
12CYS	0.00–40.00	≤8.00
12SER	0.00–40.00	≤8.00
12VAL	0.00–40.00	≤7.50
13ASP	0.00–40.00	≤7.50

* Acceptable values are within and including the values shown.

- If the FAM C_T falls within the specified range, it is FAM amplification positive.
- If the FAM C_T is above the specified range or there is no amplification, it is FAM amplification negative.

Calculate the ΔC_T value for each mutation tube that is FAM amplification positive as follows, ensuring that the mutation and control C_T values are from the same sample.

$$\Delta C_T = \text{mutation } C_T - \text{control } C_T$$

Compare the ΔC_T value for the sample with the cutoff point for the assay in question (Table 24), ensuring that the correct cutoff point is applied to each assay.

The cutoff point is the point above which a positive signal could potentially be due to background signal of the ARMS primer on wild-type DNA. If the sample ΔC_T value is higher than the cutoff point, it is classed as negative or beyond the limits of detection of the kit.

For every sample, each mutation reaction will be given a status of "mutation detected," "mutation not detected," or "invalid" using the following criteria:

Mutation detected:

- FAM amplification positive and the ΔC_T are at or below the cutoff value. If multiple mutations are detected, the mutation reported should be the one with the smallest ΔC_T value.

Mutation not detected:

- FAM amplification positive and the ΔC_T are above the cutoff value.
- FAM amplification negative and HEX (internal control) amplification positive.

Invalid:

- HEX (internal control) is invalid.
- FAM amplification negative and HEX amplification negative.

If a sample is HEX amplification negative in a tube but FAM amplification positive in a different tube, then a "mutation detected" result in the different tube can still be considered valid but the particular mutation identified may not be reliably assigned.

- If a sample is HEX amplification negative and FAM amplification positive in the same tube, then the “mutation detected” result should be considered valid.
- If a tube is HEX (internal control) invalid, the result of that tube must not be used.

Assigning sample mutation status

Once all mutation reaction tubes are assessed, the mutation status of the sample is determined as follows:

- **Mutation detected:** One or more of the 7 mutation reactions are positive. If multiple mutations are detected, the mutation reported should be the one with the smallest ΔC_T value.
- **Mutation not detected:** All 7 mutation reactions are negative.
- **Invalid:** No mutation reactions are positive, and one or more mutation reactions are invalid.

Note: The *therascreen* KRAS RGQ PCR Kit is intended to detect mutations in the KRAS gene in a DNA sample. When a sample is called KRAS mutation detected, only one specific mutation should be reported. If multiple mutations are detected, the mutation reported should be the one with the smallest ΔC_T value.

Some cross-reactivity may occur between mutation reactions. For example, if a high level 12ALA mutation is observed, some of the other mutation reactions may also give a positive result. This is due to the ARMS primers detecting other mutations of a similar sequence to one another. If a second mutation assay gives a positive result, this is likely to be cross-reactivity. Double mutants have been observed, although these are rare.

If one or more of the mutation reactions are invalid but one or more are positive, the sample can still be called KRAS mutation detected as a mutation is present. However, the specific mutation reported may not be accurate and may be a result of cross-reactivity. Therefore, the sample should only be called KRAS mutation detected.

Appendix 2: Installation of the *therascreen* KRAS Assay Package

The *therascreen* KRAS RGQ PCR Kit is designed for use with the Rotor-Gene Q MDx with a 72-well rotor. The *therascreen* KRAS Assay Package is available separately on CD (cat. no. 9022641).

The *therascreen* KRAS Assay Package is available for download on the corresponding *therascreen* KRAS RGQ PCR Kit product web page on www.qiagen.com. The download information can be found in the "Product Resources" section under the tab "Supplementary Protocols." The Assay Packages can also be ordered on a CD.

The package includes "*therascreen* KRAS CE QC Locked Template" and "*therascreen* KRAS CE Locked Template."

Note: The *therascreen* KRAS Assay Package will only work with the corresponding Rotor-Gene Q software version 2.3 with *therascreen* KRAS Assay Package version 3.1.1 (QIAGEN, cat. no. 9023675). Make sure the correct version of the Rotor-Gene Q software is installed before proceeding with the *therascreen* KRAS Assay Package installation.

Procedure (download)

1. Download the *therascreen* KRAS RGQ Assay Package on the corresponding *therascreen* KRAS RGQ PCR Kit product web page on www.qiagen.com.
2. Open the downloaded zip-file by double-clicking on the file and extracting the file inside the archive.
3. Start the installation by double-clicking the extracted file "*therascreen_KRAS_Assay_Package_3.1.1.exe*."

Procedure (CD)

1. **Order the *therascreen* KRAS RGQ Assay Package CE CD compatible to the installed Rotor-Gene Q Software (see above) available separately from QIAGEN.**

Version 3.1.1. Cat. no. 9023675.

2. Insert the CD into the CD drive of the laptop connected to the Rotor Gene Q MDx instrument.
3. Start the installation by double-clicking the file:
`therascreen_KRAS_Assay_Package_3.1.1.exe`
OR
`therascreen_KRAS_Assay_Package_1.0.12.exe`.
4. The setup wizard will appear. Click "Next" to continue (Figure 39).

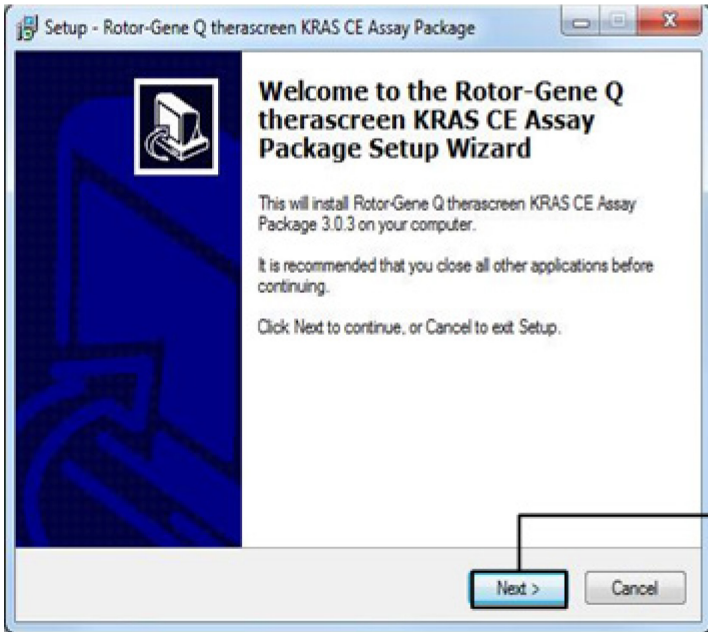


Figure 39. The "Setup" dialog box. 1 = "Next."

5. Read the License Agreement in the "License Agreement" dialog box and accept the agreement by checking the statement "I accept the agreement." Click "Next" to continue (Figure 40).

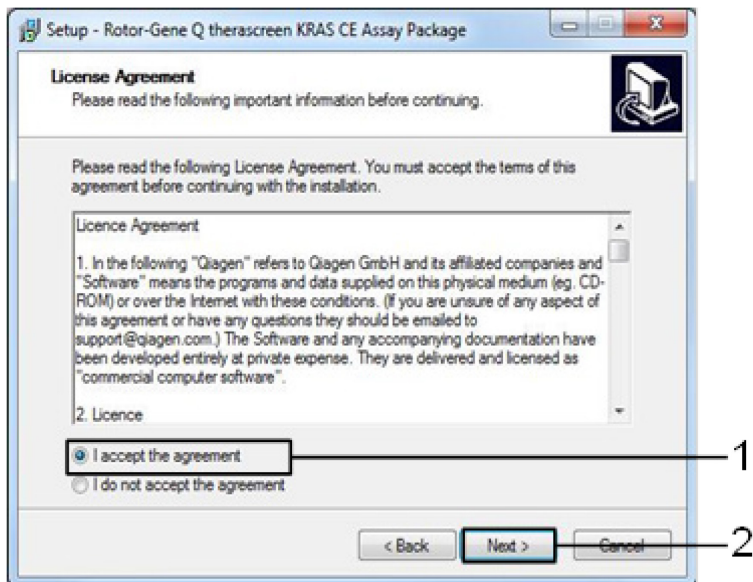


Figure 40. The "License Agreement" dialog box. 1 = "I accept the agreement" statement, 2 = "Next."

6. The template setup will start automatically, and a final “Setup” dialog box will appear. Click “Finish” to exit the setup wizard (Figure 41).

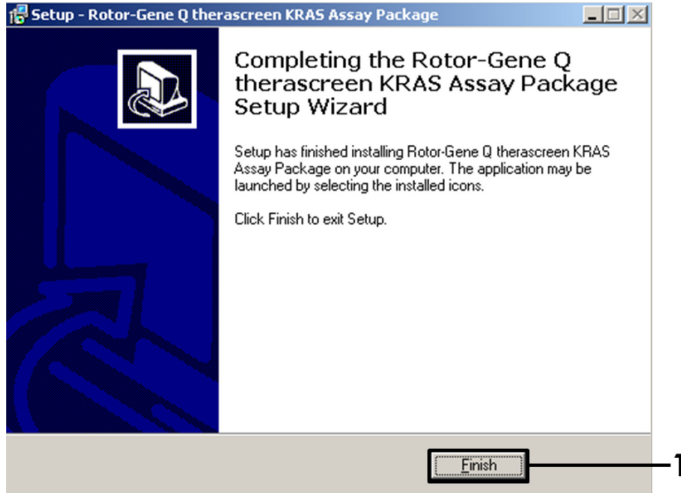


Figure 41. Completing the wizard.

7. Restart the computer. Shortcuts to both the “thetascreen KRAS QC Locked Template” and “thetascreen KRAS Locked Template” will be generated automatically and will appear on the desktop.

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> KRAS RGQ PCR Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, Water, <i>Taq</i> DNA Polymerase	874011
<i>therascreen</i> KRAS Assay Package CD (version 3.1.1)	Software protocol package for use with the <i>therascreen</i> KRAS RGQ PCR Kit and the QIAGEN Rotor-Gene Q MDx instrument with a 72-well rotor	9023675
Rotor-Gene Q and accessories		
Rotor-Gene Q MDx	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training not included	9002032
Rotor-Gene Q MDx	Real-time PCR cycler and High Resolution Melt analyzer with 5 channels (green, yellow, orange, red, crimson) plus HRM channel, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002033
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
Strip Tubes and Caps, 0.1 ml (250)	250 strips of 4 tubes and caps for 1000 reactions	981103

Strip Tubes and Caps, 0.1 ml
(2500)

10 x 250 strips of 4 tubes and caps
for 10,000 reactions

981106

**QIAamp DNA FFPE Tissue Kit — for purification of genomic DNA from
paraffin-embedded tissues**

QIAamp DNA FFPE Tissue Kit
(50)

For 50 DNA preps: QIAamp
MinElute® Columns, Proteinase K,
Buffers, and Collection Tubes (2 ml)

56404

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

Revision History

Document revision history

R4 01/2019	Addition of Authorized Representative (front cover). Updated "Symbols" section. Updated template.
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Not for use with stool samples.

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