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

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Version 1

IVD

Qualitative in vitro diagnostics

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

For use with QIAamp® DNA FFPE Tissue Kit

CE

REF   874011

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QIAGEN

Contents

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 5plex HRM 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 receptor (EGFR)-directed therapies such as panitumumab and cetuximab for the treatment of CRC. The *therascreen* KRAS RGQ PCR Kit is also intended to aid in the identification of NSCLC patients for treatment with sotorasib (LUMYKRAS®) based on a KRAS G12C mutation detected result.

Somatic mutations in the KRAS gene may also be indicated as a potential predictive biomarker to make treatment decisions for other 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.

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

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

Summary and Explanation

Mutations in the KRAS oncogene are frequently found in human cancers (1–4). Using ARMS® (Allele Refractory Mutation System) and Scorpions® 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.

The *therascreen* 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 HEX™-labeled reagents to detect internal controls. 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 5plex HRM instrument. The Rotor-Gene Q Software and the *therascreen* KRAS Assay Package is available for web download at **www.qiagen.com** or separately on CD.

- Refer to Materials Required but Not Provided, page 12, for compatible RGQ software and *therascreen* KRAS Assay Package versions.
- Refer to instrument user manual for information concerning the instrument.
- To minimize flags for the controls and samples, strict compliance to the *therascreen KRAS RGQ PCR Kit Instructions for Use* guidance should be followed with respect to placement of the Rotor-Gene Q MDx 5plex HRM instrument according to Installation Procedures and site requirements.
- Refer to Appendix 2: Installation of the therascreen KRAS Assay Package, page 134, for instructions on installing the Rotor-Gene Q *therascreen* KRAS Assay Package.

Rotor-Gene Q MDx 5plex HRM 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

therascreen 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	_Taq_	80 µl
White	Water for NTC	NTC	1.9 ml
White	Water for Sample Dilution	Dil.	1.9 ml
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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)
- 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 5plex HRM with fluorescence channels for Cycling Green and Cycling Yellow (detection of FAM and HEX, respectively)
- Rotor-Gene Q software version 2.3.1 or later with the KRAS Assay Package (version 3.0.3) installed for automated mutation detection)

  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.

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

- 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*

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.
† Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

Warnings and Precautions

For in vitro diagnostic use

For use with Rotor-Gene Q MDx 5plex HRM instrument

For use with QIAamp DNA FFPE Tissue Kit

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.

General precautions

The user should always pay attention to the following when using the *therascreen* KRAS RGQ PCR Kit:

- The test is for use with formalin-fixed, paraffin-embedded tissue specimens.
- All chemicals and biological materials are potentially hazardous. Specimens and samples are potentially infectious and must be treated as biohazardous materials.
- Discard sample and assay waste according to your local safety procedures.
- Reagents for the *therascreen* KRAS RGQ PCR Kit are diluted optimally. Do not dilute reagents further as this may result in a loss of performance. Do not use reaction volumes (reaction mix plus sample) of less than 25 µl.
- All reagents in the *therascreen* KRAS RGQ PCR Kit are formulated specifically for use with the tests provided in the kit.

- All reagents supplied in the *therascreen* KRAS RGQ PCR Kit are intended to be used solely with the other reagents in the *therascreen* KRAS RGQ PCR Kit. Do not substitute the reagents in the *therascreen* KRAS RGQ PCR Kits as this may affect performance.
- Only use *Taq* DNA polymerase (tube *Taq*) that is provided in the *therascreen* KRAS RGQ PCR 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.
- Do not use expired or incorrectly stored components.
- To minimize flags for the controls and samples, strict compliance to the *therascreen KRAS RGQ PCR Kit Instructions for Use* guidance should be followed, including but not limited to:
  ○ Correct reagent mixing is required and must be ensured at each mixing step during the assay setup.
  ○ Placement of the Rotor-Gene Q MDx 5plex HRM instrument according to the Installation Procedures and site requirements.

Note: Use extreme caution to prevent contamination of the control and reaction mix reagents with the synthetic materials that are contained in the positive control reagent.

Note: Use individual, dedicated pipets for setting up reaction mixes and adding positive control reagents.

Note: Perform preparation and dispensing of reaction mixes in an area separate from the one used for the addition of the positive control.

Note: Do not open the Rotor-Gene Q MDx 5plex HRM instrument until the run has finished.

Note: Do not open the Rotor-Gene Q tubes after the run has finished.

Note: Caution must be observed to ensure correct sample testing with emphasis to wrong sample entry, loading error, and pipetting error.

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 from CRC tissue

- 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 Hematoxilyn & 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 mm^2 in area, process 2 or more sections to increase the total tumor area to at least 4 mm^2 (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.

  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 from NSCLC tissue

- 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.

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

Specimen 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 for CRC samples

Use the QIAamp DNA FFPE Tissue Kit (QIAGEN, cat. no. 56404) with the modifications to the protocol described below for purifying genomic DNA from samples prepared from FFPE CRC specimens.

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

Carry out the DNA extraction according to the instructions in the *QIAamp DNA FFPE Tissue Kit Handbook* (Version 1) noting the following:

- See the *QIAamp DNA FFPE Tissue Kit Handbook* for preparation of specimens prior to DNA extraction.
- 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 provided in the QIAamp DNA FFPE Tissue Kit. Use only the xylene/ethanol method for deparaffinization, as described in the *QIAamp DNA FFPE Tissue Handbook*.
- Use molecular biology grade ethanol for all required steps.*
- Use 1 slide per extraction.
- 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.

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

Note: Store genomic DNA at 2–8°C for 1 week post-extraction, and then at –25 to –15°C for up to 8 weeks before use.

DNA extraction for NSCLC samples

Use the QIAamp DNA FFPE Tissue Kit (QIAGEN, cat. no. 56404) with the modifications to the protocol described below for purifying genomic DNA from samples prepared from FFPE NSCLC specimens.

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

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

- Do not use the RNase step described in the *QIAamp DNA FFPE Tissue Kit Handbook*.
- Do not use QIAGEN Deparaffinization Solution provided in the QIAamp DNA FFPE Tissue Kit. Use only the xylene/ethanol method for deparaffinization, as described in the *QIAamp DNA FFPE Tissue Handbook*.
- Use molecular biology grade ethanol for all required steps.*
- 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.

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

- 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.

Note: Store genomic DNA at 2–8°C for 1 week post-extraction, and then at –25 to –15°C for up to 8 weeks before use.

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

Note: DNA sample assessment is not designed to detect the presence of PCR inhibitors as only the total amplifiable DNA in a sample is assessed using the control reaction.

Note: It is important to use the Control Reaction Mix 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.

- Up to 24 samples can be assessed using the Control Reaction Mix (tube CTRL) available.
- Use the CTRL to assess the DNA prior to testing with the mutation assessment assay.
- 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.
- Do not vortex the *Taq* DNA polymerase (tube *Taq*) or any mix containing *Taq* DNA polymerase, as this may inactivate the enzyme.
- Pipet *Taq* DNA polymerase by carefully placing the pipet tip just under the liquid surface to avoid the tip being coated in excess enzyme.
- To minimize flags for the controls, strict compliance to the *therascreen KRAS RGQ PCR Kit Instructions for Use* guidance is required with respect to correct reagent mixing and must be ensured at each mixing step during the assay setup.

Things to do before starting

- Make sure that the Rotor-Gene Q *therascreen* KRAS Assay Package software corresponding to the Rotor-Gene software version is installed before first use of the Rotor-Gene Q MDx 5plex HRM instrument (see Appendix 2: Installation of the therascreen KRAS Assay Package).

- Before each use, all reagents need to be thawed completely for a minimum of 1 hour at room temperature (15–25°C), mixed by inverting 10 times and centrifuged briefly to collect the contents at the bottom of the tube. Correct reagent mixing must be ensured during assay setup.

- Make sure that *Taq* DNA polymerase (tube *Taq*) is at room temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

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 (15–25°C) for a minimum of 1 hour.

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

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

Thaw time		Storage* temperature after PCR setup	Maximum PCR setup and storage time
Minimum	**Maximum**		
1 hour	4.5 hours	Room temperature (15–30°C)	7 hours
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 5plex HRM instrument.

   Note: PCR setup is to be performed at room temperature.

2. 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.

   Note: Correct reagent mixing must be ensured during assay setup.

3. 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 control assay master mix contains all of the components needed for the PCR, except the sample.

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Table 3. Preparation of Control assay master mix

Component	Volume
Control Reaction Mix (CTRL)	19.76 µl x (n+1)*
Taq DNA polymerase (*Taq*)	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.

4. 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.

5. 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.

   Note: Correct reagent mixing must be ensured during assay setup.

6. 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.

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

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

9. Add 5 μl of KRAS Positive Control (PC) to the PC tube (tube position 1) and cap the tube.

   Note: Each tube should contain a total reaction volume of 25 μl (20 μl master mix prepared in Table 3, plus 5 μl of NTC/sample/PC).

10. 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 5plex HRM instrument.

11. Invert capped tubes 4 times to mix the sample and reaction mix.

    Note: Correct reagent mixing must be ensured during assay setup.

12. 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 5plex HRM instrument is maintained.

13. Place the 72-well rotor into the Rotor-Gene Q MDx 5plex HRM instrument. Make sure the locking ring (supplied with the Rotor-Gene Q MDx 5plex HRM instrument) is placed on top of the rotor to secure the tubes during the run.

14. Double-click the **therascreen KRAS QC Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q MDx 5plex HRM instrument (Figure 1) to start the Rotor Gene Q software.

Figure 1. The therascreen KRAS QC Locked Template icon.

The Setup tab appears as the default (Figure 2).
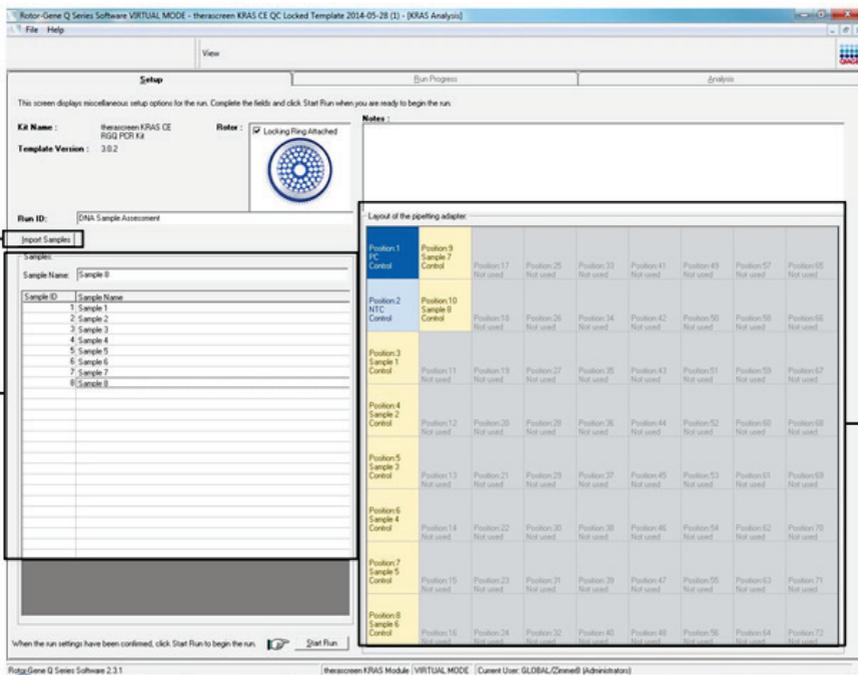
Figure 2. The Setup tab and Locking Ring Attached box. 1 = Setup tab, 2 = Locking Ring Attached box.

15. Ensure that the locking ring is properly attached and check the **Locking Ring Attached** box. Close the lid of the Rotor-Gene Q MDx 5plex HRM instrument.

16. Enter the run ID in the **Run ID** field according to your local naming convention. Enter the sample name in the **Sample Name** 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 completely display in the Layout of the pipetting adapter panel.
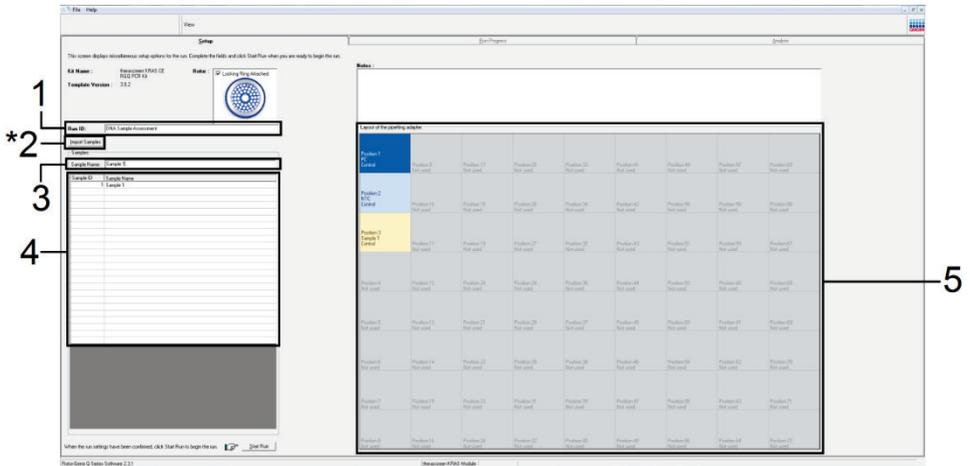
Figure 3. Entering the Run ID and Sample Name. 1 = Run ID dialog field, 2 = Import Sample button 3 = Sample Name dialog field, 4 = Sample List, 5 = Layout of the pipetting adapter panel.

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

    Note: To edit a sample name, click **Sample Name** in the sample list and the selected sample will appear in the **Sample Name** field above. Edit the sample name according to your local naming convention and press the **Return** key to update the name.
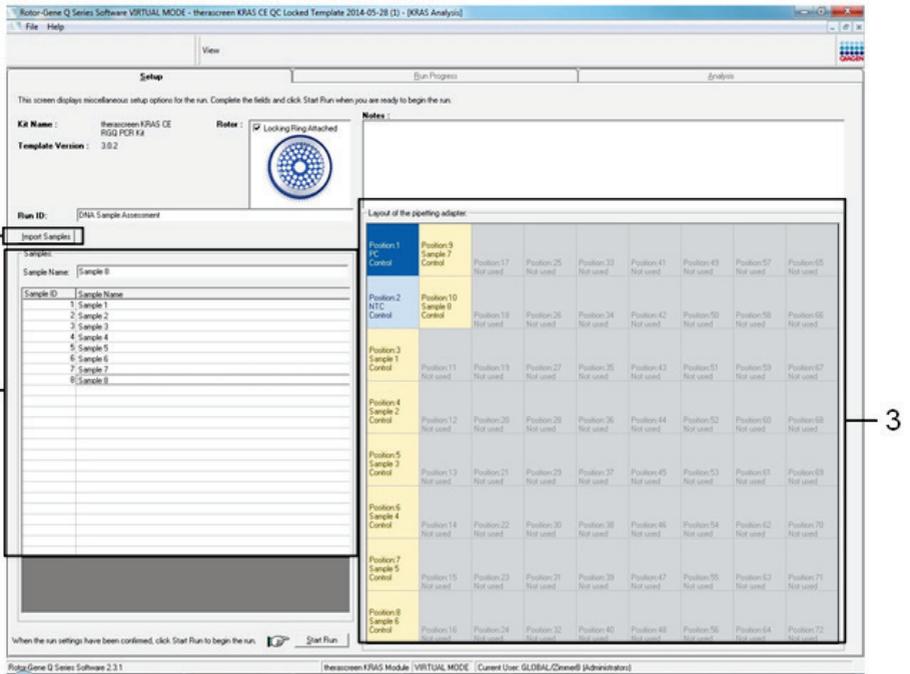
Figure 4. Entering additional sample names in the Sample Name dialog field. *1 = Import Sample button, 2 = Sample Name dialog field and Sample List, 3 = Layout of the pipetting adapter panel with additional sample name.

18. When all sample names have been entered, verify that they are correct. Add any additional information in the **Notes** field if necessary, then click **Start Run** (Figure 5).

    Note: If any rotor position is unused, a warning message 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.
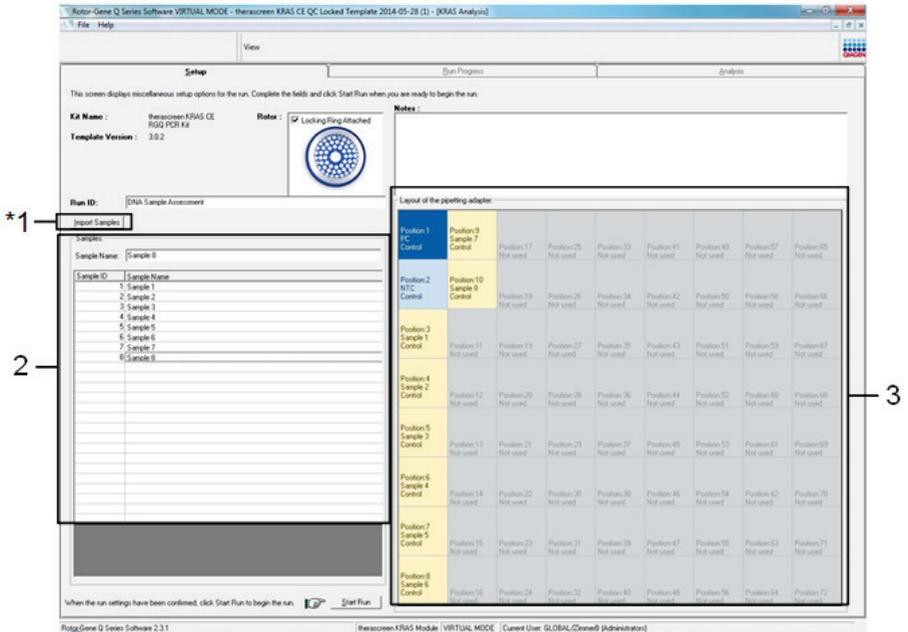
Figure 5. Notes dialog field, Start Run, and Warning of unused rotor positions.
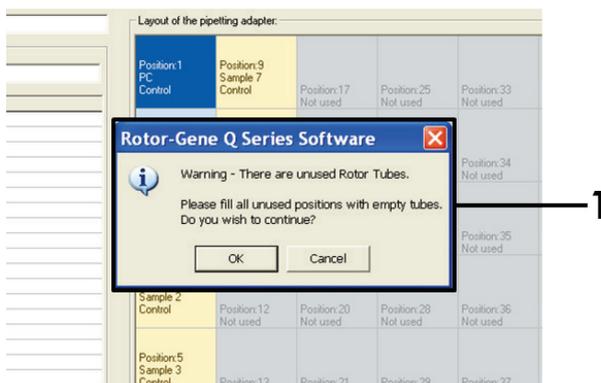
Figure 6. 1 = Warning of unused rotor positions.

19. A Save As dialog box appears. Select an appropriate file name and save the PCR run as a *.rex run file to the selected location. Click **Save** (Figure 7).
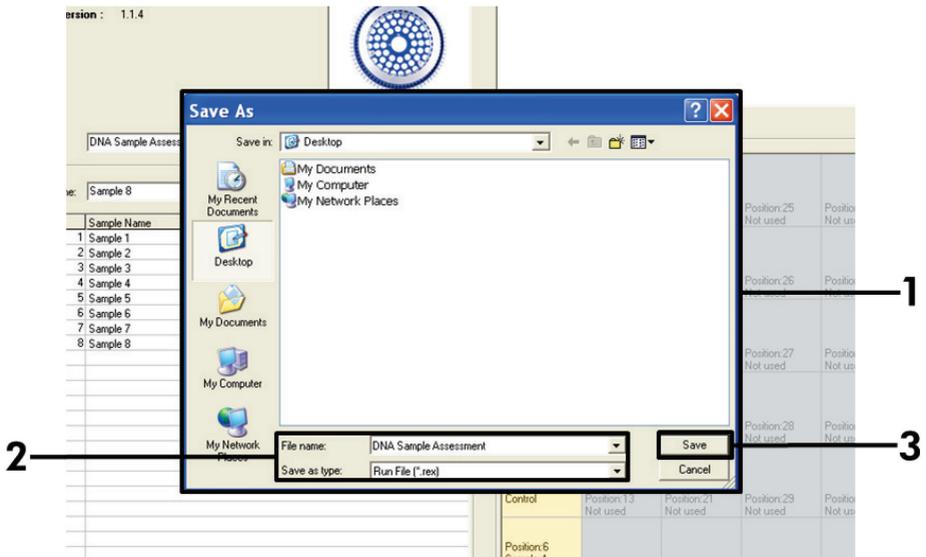
Figure 7. Saving the run file. 1 = Save As window, 2 = File name and save as type *.rex file, 3 = **Save**.

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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).
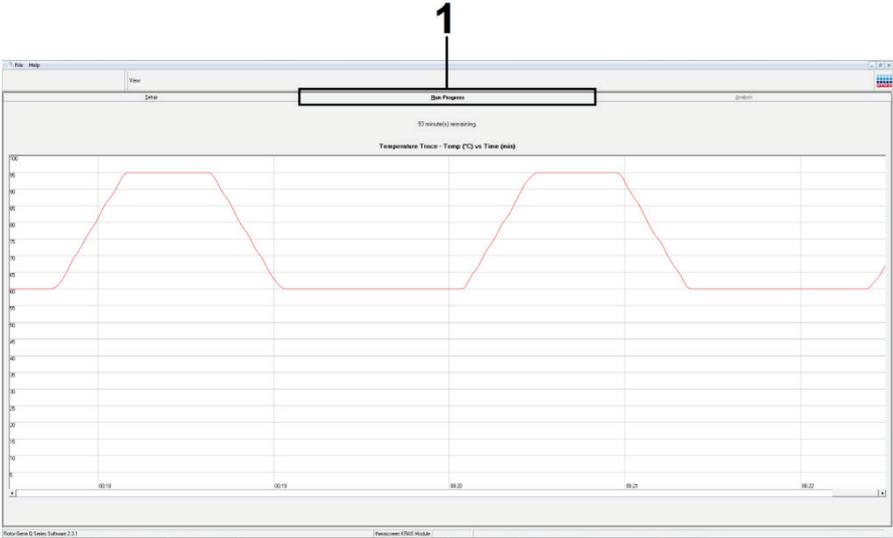
Figure 8. The Run Progress tab.

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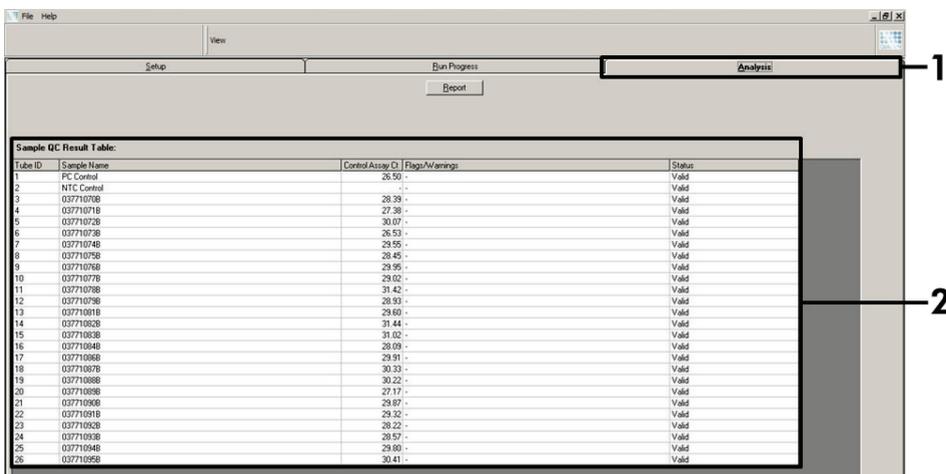
Figure 9. The Analysis tab and reporting of results. 1 = Analysis tab, 2 = Sample QC Result Table.

Note: 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 Interpretation of Results (Manual).

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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%** in the **NTC Threshold** field.

9. Set the threshold at **0.05** and check the HEX CT values.

10. On the analysis page, click **Cycling A. Green** to view the FAM channel.

11. Verify that **Dynamic Tube** is highlighted. Click **Linear Scale**.

12. Click **Outlier Removal** and enter **10%** in the **NTC Threshold** field.

13. Set the threshold at **0.05** and check the FAM CT values.

Sample assessment data analysis

Run control analysis

Refer to the Run control analysis flowchart in Figure 42.

- **Negative control:** To ensure that 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.

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KRAS mutation detection analysis

Run control analysis

Refer to the Run control analysis flowchart (Figure 10).

- **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.
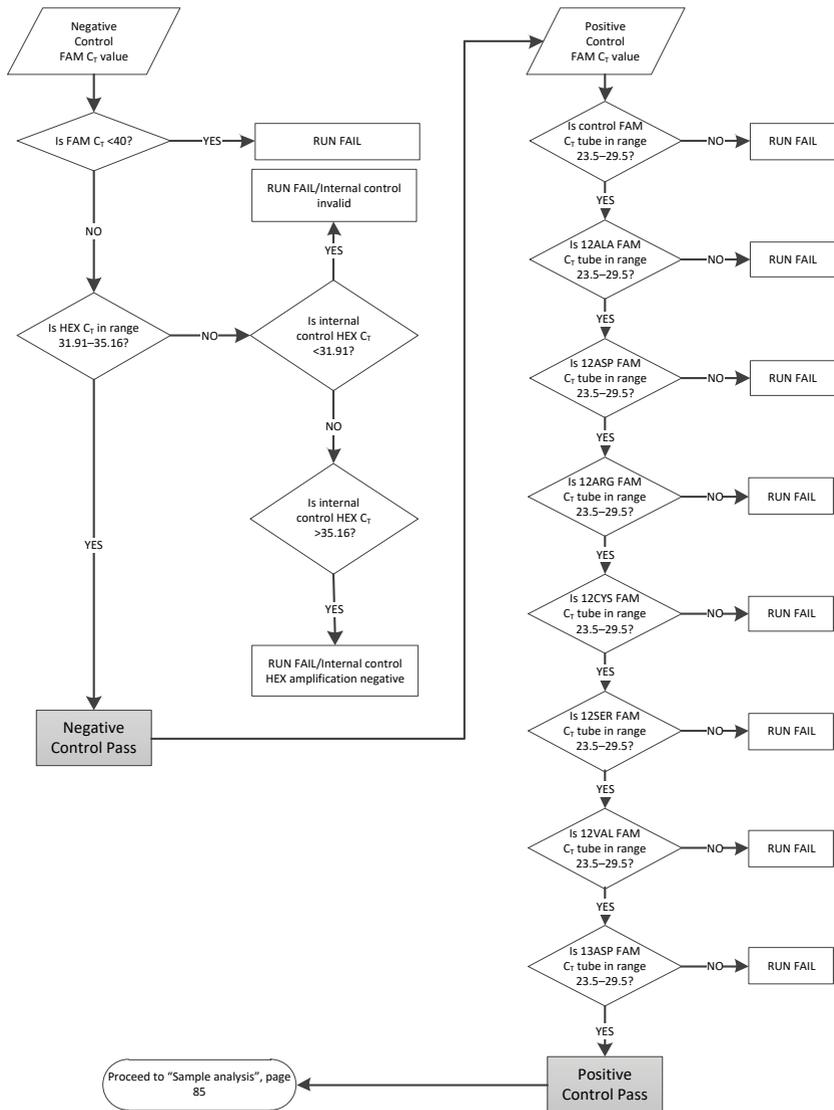
Figure 10. Run control analysis flowchart.

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Sample analysis

Refer to the Sample analysis flowchart in Figure 11.

Sample control FAM CT 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.
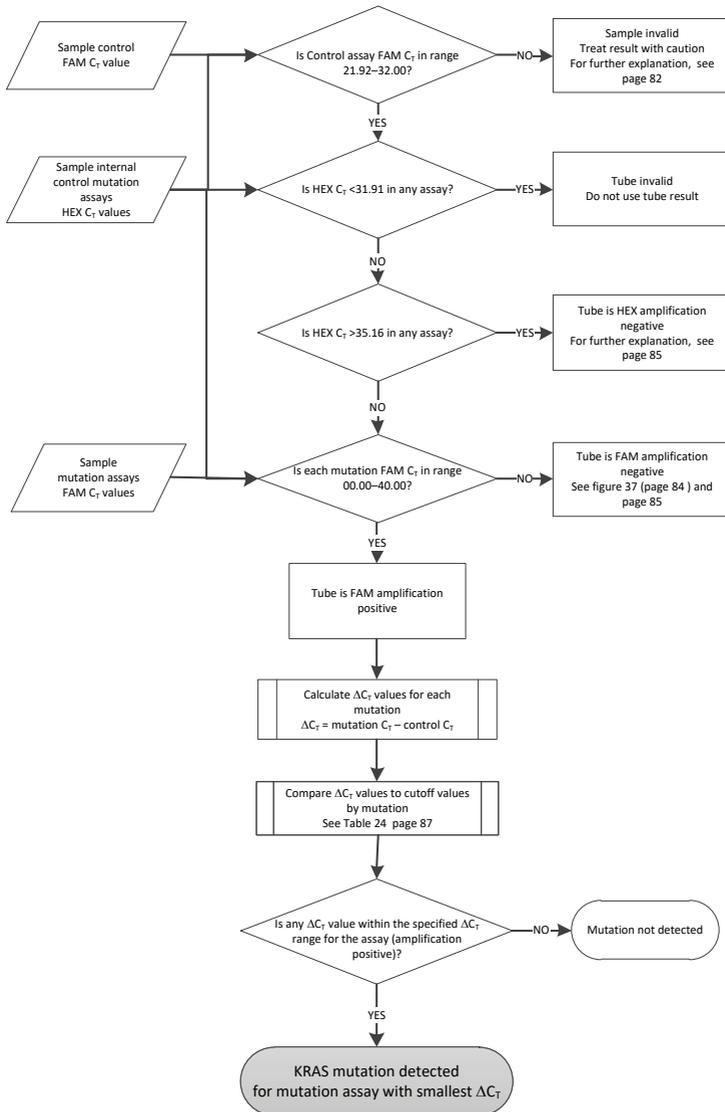
Figure 11. Sample analysis flowchart.

Sample internal control mutation assays HEX CT value

Refer to the Sample analysis flowchart in Figure 11.
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 CT value

The FAM values for all 7 reaction mixes should be checked against the values listed in Table 5.

Table 5. 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 CT falls within the specified range, it is FAM amplification positive.
- If the FAM CT is above the specified range or there is no amplification, it is FAM amplification negative.
- Calculate the DCT value for each mutation tube that is FAM amplification positive as follows, ensuring that the mutation and control CT 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 21), 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 DCT are at or below the cutoff value. If multiple mutations are detected, the mutation reported should be the one with the smallest DCT value.

Mutation not detected:

- FAM amplification positive and the DCT 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 DCT 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.

- **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 ≤ Control C_T ≥ 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.

14. To generate report files, click **Report**. The Report Browser window will appear. Select **KRAS Analysis Report** under Templates, then click **Show** (Figure 12).

    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.
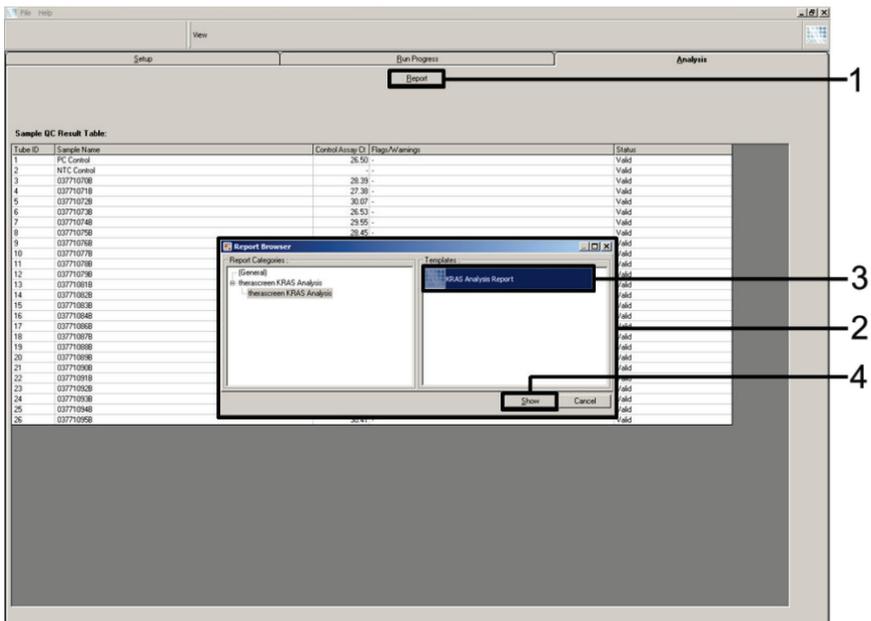
Figure 12. Selecting the KRAS Analysis Report. 1 = Report, 2 = Report Browser window, 3 = KRAS Analysis Report selection, 4 = Show.

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.

- The sample must be tested using all reaction mixes provided in the *therascreen* KRAS RGQ PCR Kit.

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

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

- To minimize flags for the controls and samples, strict compliance to the *therascreen KRAS RGQ PCR Kit Instructions for Use* guidance is required with respect to correct reagent mixing and must be ensured at each mixing step during the assay setup.

- 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 5plex HRM instrument.

Things to do before starting

- Before each use, all reagents need to be thawed completely for a minimum of 1 hour at room temperature (15–25°C), mixed by inverting 10 times, and centrifuged briefly to collect the contents at the bottom of the tube.

- Ensure that *Taq* DNA polymerase (tube *Taq*) is at room temperature (15–25°C) before each use. Centrifuge the tube briefly to collect the enzyme at the bottom of the tube. Correct reagent mixing must be ensured during assay setup.

Procedure

1. Completely thaw all reaction mix tubes, nuclease-free water for no template control tube (NTC) and KRAS Positive Control (tube PC) at room temperature (15–25°C) for a minimum of 1 hour.

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

Table 5. Time for thawing reagents

Thaw time			
Minimum	Maximum	Storage temperature after PCR setup	Maximum PCR setup and storage time
1 hour	4.5 hours	Room temperature (15–25°C)	7 hours
1 hour	4.5 hours	2–8°C	18 hours

   Note: PCR setup is to be performed at room temperature. The term "storage" refers to the time between completion of PCR setup and start of the PCR run on the Rotor-Gene Q MDx instrument.

   Note: Bring *Taq* DNA polymerase (tube Taq) to room temperature (15–25°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.

2. When the reagents have thawed, mix them by inverting each tube 10 times to avoid localized concentrations of salts and then centrifuge directly to collect the contents at the bottom of the tube.

   Note: Correct reagent mixing must be ensured during assay setup.

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3. Label 8 microcentrifuge tubes (not provided) according to each corresponding reaction mix shown in the table below. Prepare sufficient master mixes (control or mutation reaction mix [tube CTRL, 12ALA, 12ASP, 12ARG, 12CYS, 12SER, 12VAL, or 13ASP] plus *Taq* DNA polymerase [Taq]) for the DNA samples, one KRAS Positive Control (tube PC) reaction, and one nuclease-free water for no template control (tube NTC) reaction according to the volumes in the table. Include reagents for 1 extra sample to allow sufficient overage for the PCR setup.

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

Table 6. Master mix and corresponding volume

Assay and reaction mix tube	Volume of reaction mix	Volume of *Taq* DNA polymerase
Control (tube CTRL)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
12ALA (tube 12ALA)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
12ASP (tube 12ASP)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
12ARG (tube 12ARG)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
12CYS (tube 12CYS)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
12SER (tube 12SER)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
12VAL (tube 12VAL)	19.76 µl x (n + 1)	0.24 µl x (n + 1)
13ASP (tube 13ASP)	19.76 µl x (n + 1)	0.24 µl x (n + 1)

* n = number of reactions (samples plus controls).
  Prepare enough master mix for 1 extra sample (n + 1) to allow for sufficient overage for the PCR setup. The value n should not exceed 7 (plus controls) as 7 is the maximum number of samples which can fit on a run.

Note: When preparing the assay master mixes, the required volume of the control or mutation reaction mix is added to the relevant tube first and the Taq DNA polymerase (tube Taq) is added last.

4. Place the appropriate number of PCR 4-strip tubes (each strip has 4 tubes) in the loading block according to the layout in the Table 4. Run layout in the loading block for DNA sample assessment. Numbers denote positions in the loading block and indicate final rotor position. Do not cap the tubes.

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

5. 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.

   Note: Correct reagent mixing must be ensured during assay setup.

   For detection of KRAS mutations, assay master mixes should be added to 8 PC tubes, 8 NTC tubes, and 8 tubes for each DNA sample.

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

   Note: See Table 7 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 7. Run layout in the loading block for detection of KRAS mutations

Assay	Controls 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

(Controls: PC, NTC; Sample number: 1–7)

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

7. 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.

8. Add 5 µl of each DNA sample to the sample tubes (tube positions 17–72) and cap the tubes.

9. Add 5 µl of KRAS Positive Control (PC) to the PC tubes (tube positions 1–8) and cap the tubes.

10. 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 5plex HRM instrument.

11. Invert the capped tubes 4 times to mix the sample and reaction mix.

    Note: Correct reagent mixing must be ensured during assay setup.

12. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 7) 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 5plex HRM instrument is maintained.

13. Place the 72-well rotor into the Rotor-Gene Q MDx 5plex HRM instrument. Ensure that the locking ring (supplied with the Rotor-Gene Q MDx 5plex HRM instrument) is placed on top of the rotor to secure the tubes during the run.

14. Double-click the **therascreen KRAS Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q MDx 5plex HRM instrument (Figure 13) to start the Rotor Gene Q MDx 5plex HRM software.
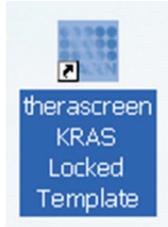
Figure 13. The therascreen KRAS Locked Template icon.

The Setup tab appears as the default (Figure 14).
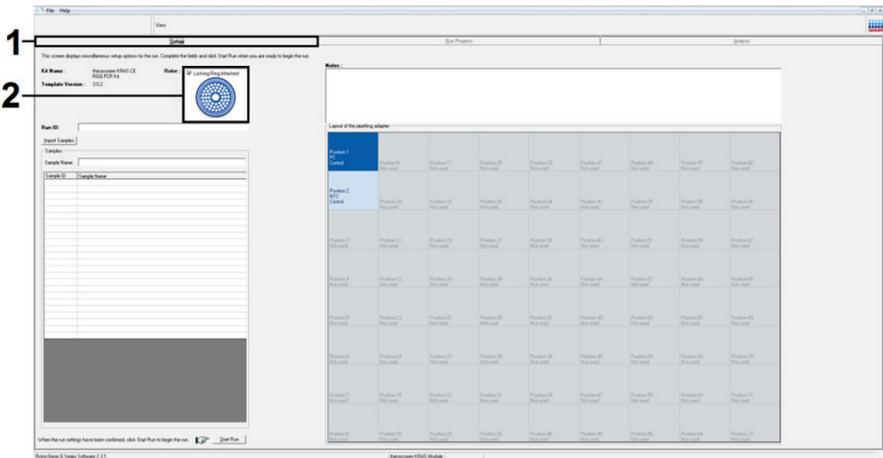
Figure 14. 1 = The Setup tab and 2 = Locking Ring Attached box.

15. Ensure that the locking ring is properly attached and check the **Locking Ring Attached** box. Close the lid of the Rotor-Gene Q MDx 5plex HRM instrument.

16. Enter the run ID in the **Run ID** field according to your local naming convention.

17. Enter the sample name in the **Sample Name** 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 15).

    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 15).

    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.
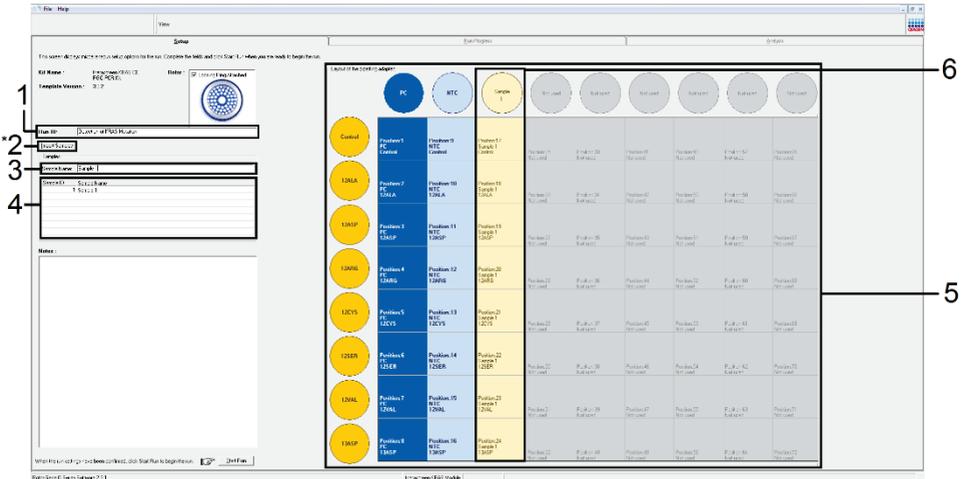
Figure 15. Entering the Run ID and Sample Name. 1 = Run ID dialog field, 2 = Import Sample (not available with software version 2.1), 3 = Sample Name dialog field, 4 = Sample List, 5 = Layout of the pipetting adapter panel, 6 = Highlighted sample circle and column of 8 assays underneath.

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18. Repeat step 14 to enter the names of all additional samples (Figure 16).

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** field above. Edit the sample name according to your local naming convention and press the **Return** key to update the name.
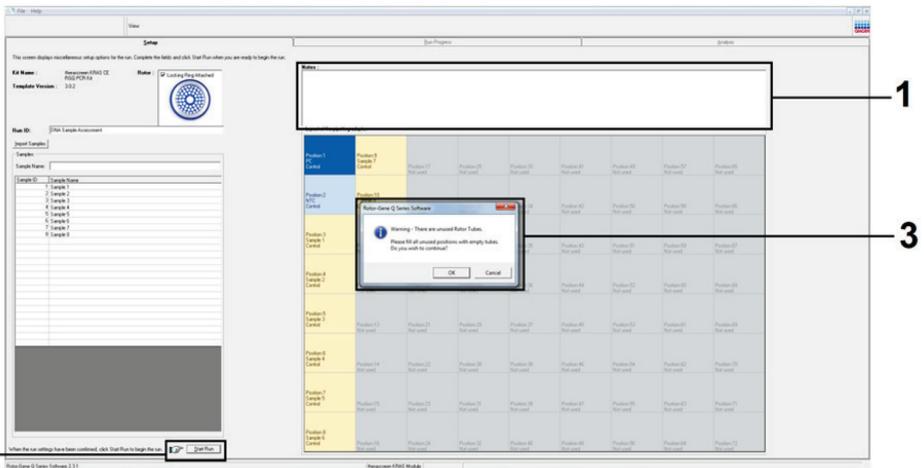
Figure 16. Entering additional sample names in the Sample Name dialog field. 1 = Sample Name dialog field, 2 = Sample List, 3 = Layout of the pipetting adapter panel with additional sample names.

19. When all sample names have been entered, verify that they are correct. Add any additional information in the **Notes** field if necessary, then click the **Start Run** (Figure 17).

Note: If any rotor position is unused, a warning message will appear (Figure 17 and Figure 18) 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.
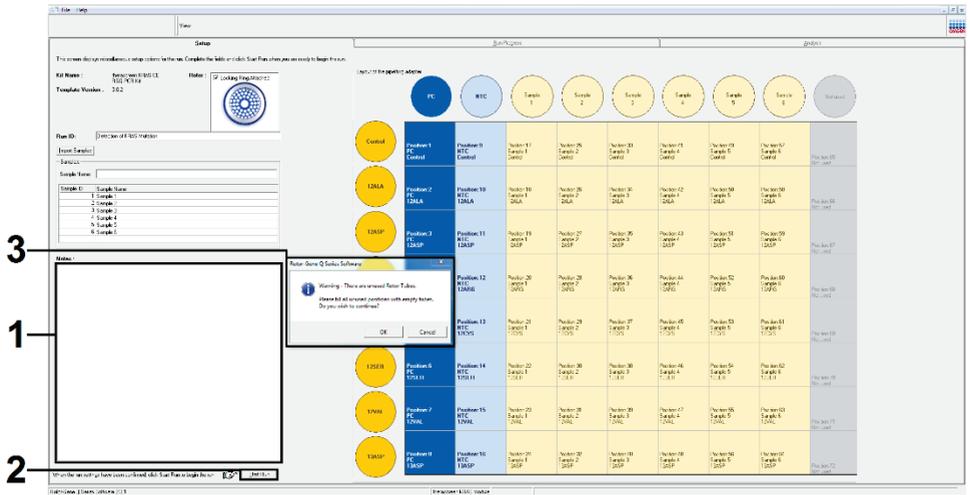
Figure 17. 1 = Notes dialog field, 2 = Start Run, and 3 = Warning of unused rotor positions.
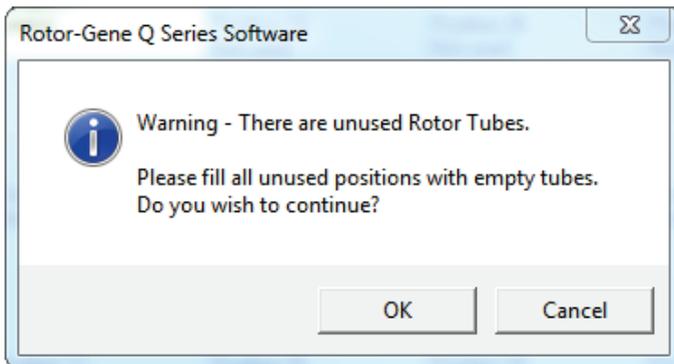
Figure 18. Warning of unused rotor positions.

20. In the Save As window, select an appropriate file name and save the PCR run as a
    *.rex run file to the selected location (Figure 19).
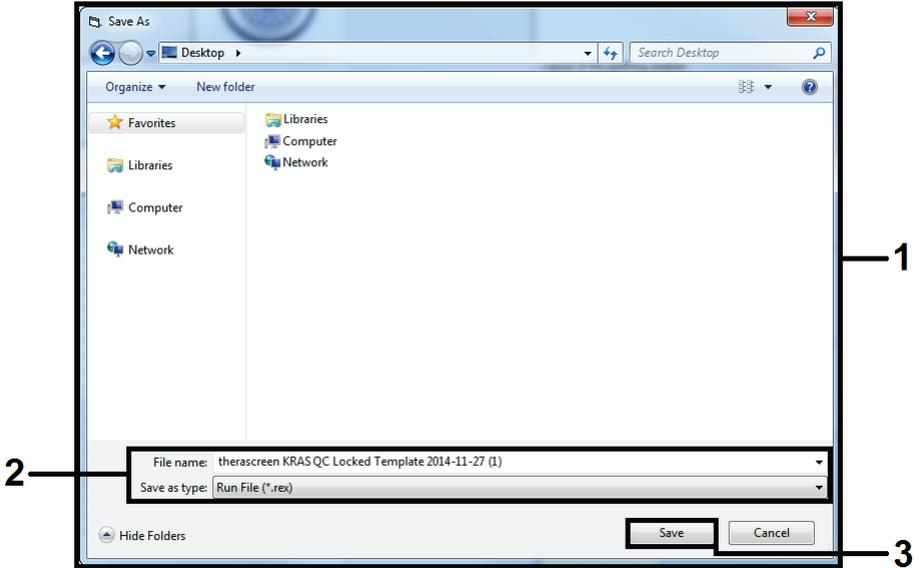
Figure 19. Saving the run file.

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 20).
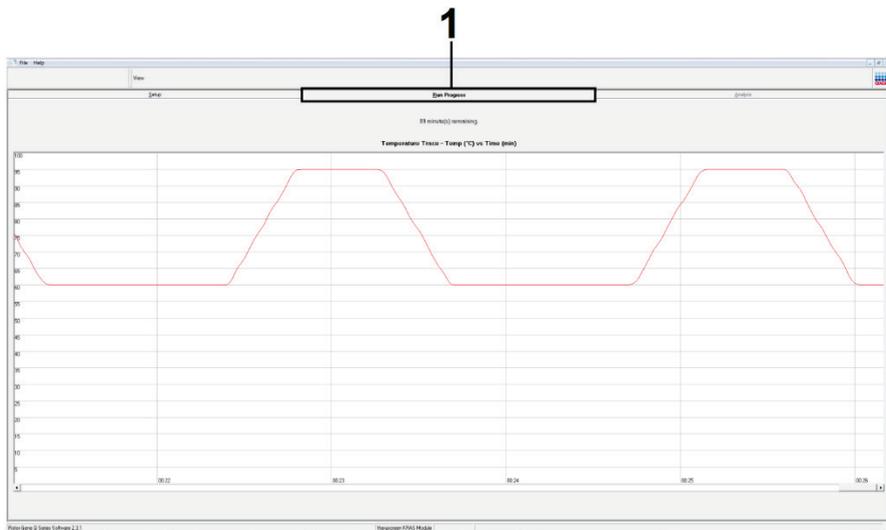
Figure 20. Run Progress tab.

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

Note: If the Analysis tab fails to open, click the Analysis tab (Figure 21).

Note: An explanation of the calculation method is presented in the Interpretation of Results section.
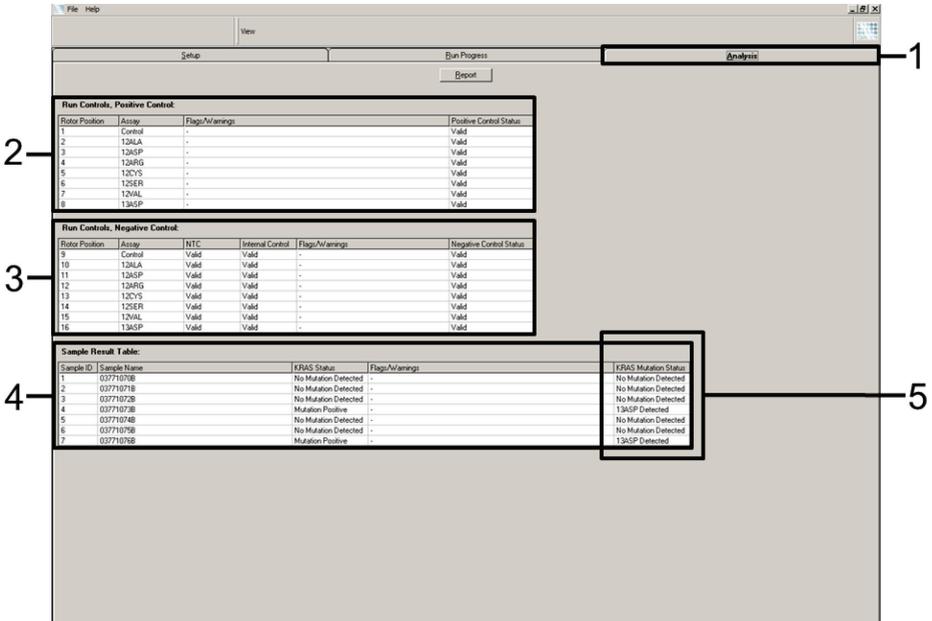
Figure 21. The Analysis tab and reporting of results. 1 = Analysis tab, 2 = Run Controls, Positive Control panel, 3 = Run Controls, Negative Control panel, 4 = Sample Result Table, 5 = KRAS Mutation Status column.

Assay results will be reported as follows (Figure 21).

- **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. To generate report files, click **Report**. The Report Browser window will appear. Select **KRAS Analysis Report** under Templates, then click **Show** (Figure 22).

   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.
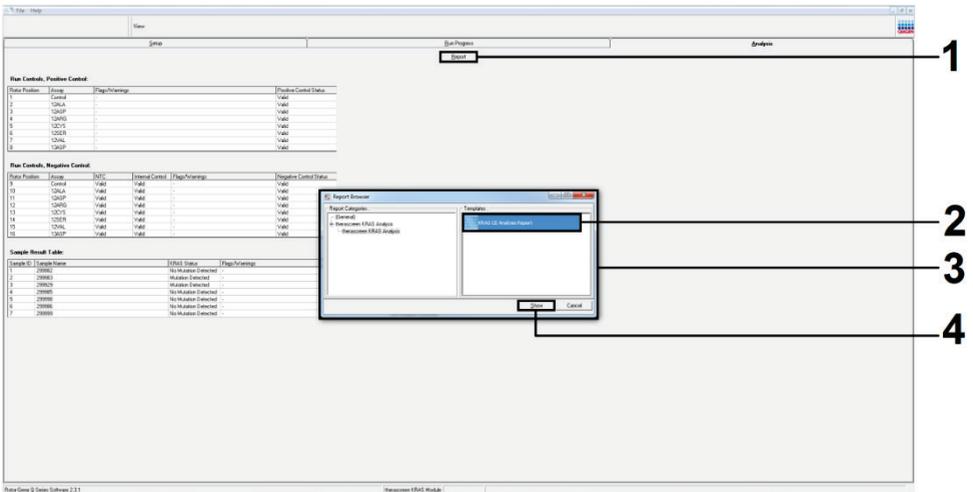
Figure 22. Selecting the KRAS Analysis Report. 1 = Report, 2 = Report Browser window, 3 = KRAS Analysis Report selection, 4 = Show.

Note for NSCLC samples only: In order to avoid calling of a false G12C (12CYS) mutation result, sample(s) with the flags listed below must be interpreted as Invalid.

- SAMPLE_INT_CTRL_EARLY_CT
- SAMPLE_POSITIVE_AND_INVALID
- SAMPLE_INT_CTRL_FAIL
- MUTATION_EARLY_CT
- SAMPLE_INVALID_DATA

Interpretation of Results

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

Analysis and mutation calls

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 name] Detected", No Mutation Detected, Invalid, or, if a run control fails, Run Control Failed. For the mutation-positive samples, specific mutations will be reported.

For interpretation of flags generated by the Rotor-Gene Q *therascreen* KRAS Assay Package, see Flags generated by the *therascreen* KRAS Assay Package.

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

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 not to be used for diagnosis of any disease.

For CRC samples, the *therascreen* KRAS RGQ PCR Kit is only intended to discriminate between wild-type and mutant samples. 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 formalin-fixed, paraffin-embedded colorectal cancer tissue and non-small cell lung cancer 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 5plex HRM 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 8.

Table 8. Established cutoff values for each mutation assay.

	Mutation assay						
	12ALA	**12ASP**	**12ARG**	**12CYS**	**12SER**	**12VAL**	**13ASP**
Cutoff ($\leq DC_T$)	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 are shown in Table 9. Table 10 shows the analysis of agreement the *therascreen* KRAS RGQ PCR Kit and bi-directional sequencing.

Table 9. *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
***therascreen* 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 10. 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 12 and Table 13).

Table 11. *therascreen* KRAS RGQ PCR Kit vs. bi-directional Sanger sequencing (second study)

		Mutation call by bi-directional sequencing									
		Neg.	12ALA	12ALA_12CYS	12ARG	12ASP	12CYS	12SER	12VAL	13ASP	Total
therascreen KRAS RGQ PCR Kit call	Negative	261	–	–	–	–	–	–	–	1	**262**
	Positive 12ALA	1	4	1	–	–	–	–	–	–	**6**
	Positive 12ARG	–	–	–	3	–	–	–	–	–	**3**
	Positive 12ASP	4	–	–	–	14	–	–	–	–	**18**
	Positive 12CYS	6	–	–	–	–	35	–	–	–	**41**
	Positive 12SER	2	–	–	–	–	–	–	–	–	**2**
	Positive 12VAL	5	–	–	–	–	–	1	17	–	**23**
	Positive 13ASP	1	–	–	–	–	–	–	–	4	**5**
	Total	280	4	1	3	14	35	1	17	5	**360**

Table 12. Analysis of agreement (second study)

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

Comparison to analytical reference method: NSCLC

To demonstrate the concordance in overall 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 were used during this study. 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 . 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 14).

Table 13. *therascreen* KRAS RGQ PCR Kit vs. bi-directional Sanger sequencing

		Mutation call by bi-directional sequencing									
		Neg.	12ALA	12ALA_12CYS	12ARG	12ASP	12CYS	12CYS_12VAL	12VAL	13ASP	Total
therascreen KRAS RGQ PCR Kit call	Negative	261	–	–	–	–	–	–	–	1	**262**
	Positive 12ALA	1	4	1	–	–	–	–	–	–	**6**
	Positive 12ARG	–	–		3	–	–	–	–	–	**3**
	Positive 12ASP	4		-		14					**18**
	Positive 12CYS	6	–	-	–	–	35	–	–	–	**41**
	Positive 12SER	2	–	-	–	–	–	–	–	–	**2**
	Positive 12VAL	5	–	-	–	–	–	1	17	–	**23**
	Positive 13ASP	1	–	-	–	–	–	–	–	4	**5**
	Total	**280**	**4**	**1**	**3**	**14**	**35**	**1**	**17**	**5**	**360**

Table 14. 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 16).

Table 15. LOD values for each mutation assay using FFPE cell lines

Assay	LOD C$_{95}$ (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. The objective of the study was to demonstrate that the performance of the *therascreen* KRAS RGQ PCR Kit is consistent over the total DNA input (control CT) range of the assay. DNA extracted from 8 FFPE cell lines was used to prepare pools of DNA with the lowest achievable control reaction C_T. Concentrated DNA stocks were subsequently diluted to generate DNA spanning the working range (a total of 5 dilutions including the initial concentrated stock).

For each point within the working range, sufficient material was prepared to carry out 6 replicate tests. The dilution range for each mutation reaction and the mean ΔC_T value obtained from the results are shown in Table and Table . 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 16. 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 17. 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 [10(–1/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 18 (CRC samples) and Table 19 (NSCLC samples).

Table 18. 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**	

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Table 19. 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**	

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 22–23C_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 23 C_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.

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 5plex HRM 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 5plex HRM 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 and Table 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 20. Cross-reactivity (ΔCT) 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 21. Cross-reactivity (ΔCT) 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 precision of the *therascreen* KRAS RGQ PCR Kit was determined using a protocol incorporating aspects of CLSI EP12-A and EP5-A2 (21, 22). 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 (3xLOD) 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 experiments. Estimates of variance demonstrated (1x standard deviation) using C50 and 3xLOD samples are listed in Table 23 and Table 24.

Table 22. Assay variance estimates

Assay	%CV for ΔC_T		%CV for mutant C_T		%CV for control C_T		
	3xLOD	C50	3xLOD	C50	3xLOD	C50	WT
12ALA	13.14	8.32	1.87	2.02	0.97	1.12	1.12
12ARG	10.79	8.04	1.59	1.96	1.24	1.51	1.15
12ASP	12.86	5.87	1.11	1.00	0.90	0.90	1.04
12CYS	17.61	10.83	1.86	2.02	1.54	1.22	1.15
12SER	13.97	10.43	1.71	2.11	0.94	1.19	1.15
12VAL	9.66	15.47	1.52	1.65	1.11	3.74	1.26
13ASP	13.73	9.35	1.91	2.08	1.11	1.41	1.19

Table 23. Repeatability precision estimates

Assay	%CV for ΔC_T		%CV for mutant C_T		%CV for control C_T		
	3xLOD	C50	3xLOD	C50	3xLOD	C50	WT
12ALA	10.71	7.51	1.69	1.76	0.77	0.90	0.79
12ARG	9.83	8.04	1.21	1.76	0.84	1.33	0.90
12ASP	10.16	4.08	0.93	0.89	0.80	0.76	0.76
12CYS	13.15	8.80	1.31	1.76	1.40	1.01	0.76
12SER	6.76	6.18	1.10	1.48	0.80	0.90	0.90
12VAL	9.21	15.32	1.40	1.42	0.91	3.49	0.94
13ASP	8.67	7.01	1.30	1.65	0.91	1.19	0.97

The estimated proportion of 3xLOD samples testing mutant and wild-type samples were 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. The overall proportion of correct calls

was 99.6% (1115/1120); 99.6% (558/560) for mutation positive (3xLOD) samples and 99.5% (557/560) for no-mutation detected (wild-type) samples (Table 25).

Table 24. Correct calls overall

| Mutation | Correct calls | |
	3xLOD samples	Wild-type samples (low)
12ALA	79/80	80/80
12ARG	80/80	79/80
12ASP	80/80	80/80
12CYS	79/80	80/80
12SER	80/80	79/80
12VAL	80/80	79/80
13ASP	80/80	80/80

NSCLC

The precision of the *therascreen* KRAS RGQ PCR Kit within-laboratory (repeatability) was assessed. Both the correctness of mutation results and the precision of ΔC_T values (the difference in C_T values between a Mutation Reaction and the Control Reaction) are reported.

In total, 15 panel members were prepared; one for each of the 7 mutations detected by the KRAS kit (at LOD and 2xLOD), and a wild type (WT) panel member. Mutant panel members were represented by either an FFPE cell line or a clinical sample depending on availability. All samples were normalized to a control C_T of 27 and mutant samples were diluted in wild type DNA to generate sufficient material for samples at mutation levels of 1x LOD and 2x LOD.

The proportion of correct calls is presented in Table 26 for each test panel and the quantitative precision values are presented in Table 27.

Table 25. Proportion of correct mutation calls

Grouping variables		Proportion		Two-sided 95% confidence limit	
Sample level	Assay	Fraction	Percentage	Lower	Upper
2xLOD	12ALA	28/28	100.00%	87.66%	100.00%
	12ARG*	28/28	100.00%	87.66%	100.00%
	12ASP	28/28	100.00%	87.66%	100.00%
	12CYS	28/28	100.00%	87.66%	100.00%
	12SER*	28/28	100.00%	87.66%	100.00%
	12VAL	28/28	100.00%	87.66%	100.00%
	13ASP*	28/28	100.00%	87.66%	100.00%
LOD	12ALA	39/40	97.50%	86.84%	99.94%
	12ARG	40/40	100.00%	91.19%	100.00%
	12ASP	40/40	100.00%	91.19%	100.00%
	12CYS	40/40	100.00%	91.19%	100.00%
	12SER*	40/40	100.00%	91.19%	100.00%
	12VAL	40/40	100.00%	91.19%	100.00%
	13ASP*	38/40	95.00%	83.08%	99.39%
WT	All	28/28	100.00%	87.66%	100.00%

* Represented by FFPE cell line

Table 26. Variance components in terms of SD and %CV – repeatability

Analysis variable	Sample level	Assay	# of observations	Mean	Bet. Day*	Bet. Run N*	Bet. Rgq*	Bet. Kit lot*	Bet. Operator*	Residual*	Total
Delta C$_T$	2xLOD	12ALA	28	5.54	(0.0000, 0.00%)	(0.1221, 2.20%)	(0.0443, 0.80%)	(0.0385, 0.70%)	(0.0000, 0.00%)	(0.1335, 2.41%)	(0.1843, 3.33%)
		12ARG	28	4.80	(0.0000, 0.00%)	(0.1891, 3.94%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.3244, 6.76%)	(0.3737, 7.79%)
		12ASP	28	4.72	(0.0860, 1.82%)	(0.1446, 3.06%)	(0.0000, 0.00%)	(0.1463, 3.10%)	(0.1374, 2.91%)	(0.1751, 3.71%)	(0.2797, 5.93%)
		12CYS	28	5.66	(0.0563, 0.99%)	(0.0000, 0.00%)	(0.0995, 1.76%)	(0.0000, 0.00%)	(0.0390, 0.69%)	(0.2306, 4.08%)	(0.2498, 4.41%)
		12SER	28	5.36	(0.1429, 2.67%)	(0.0274, 0.51%)	(0.0000, 0.00%)	(0.0647, 1.21%)	(0.0000, 0.00%)	(0.1753, 3.27%)	(0.2129, 3.97%)
		12VAL	28	4.26	(0.0000, 0.00%)	(0.1016, 2.39%)	(0.0593, 1.39%)	(0.1128, 2.65%)	(0.0000, 0.00%)	(0.2095, 4.92%)	(0.2457, 5.77%)
		13ASP	28	5.23	(0.0000, 0.00%)	(0.2892, 5.53%)	(0.0157, 0.30%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2171, 4.15%)	(0.3575, 6.83%)
	LOD	12ALA	40	6.36	(0.0000, 0.00%)	(0.1584, 2.49%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2346, 3.69%)	(0.2819, 4.43%)
		12ARG	40	5.45	(0.0036, 0.07%)	(0.1639, 3.01%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0797, 1.46%)	(0.1674, 3.07%)	(0.2397, 4.40%)
		12ASP	40	4.73	(0.0000, 0.00%)	(0.2485, 5.25%)	(0.1087, 2.30%)	(0.0000, 0.00%)	(0.0816, 1.72%)	(0.1041, 2.20%)	(0.2837, 6.00%)
		12CYS	40	6.62	(0.1688, 2.55%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2056, 3.11%)	(0.0000, 0.00%)	(0.2909, 4.40%)	(0.3652, 5.52%)
		12SER	40	6.37	(0.1006, 1.58%)	(0.3153, 4.95%)	(0.0000, 0.00%)	(0.0340, 0.53%)	(0.0000, 0.00%)	(0.2253, 3.54%)	(0.3854, 6.05%)
		12VAL	40	5.13	(0.2874, 5.60%)	(0.0976, 1.90%)	(0.0227, 0.44%)	(0.0874, 1.71%)	(0.0000, 0.00%)	(0.1629, 3.18%)	(0.2965, 5.78%)
		13ASP	38	6.26	(0.3433, 5.48%)	(0.1227, 1.96%)	(0.0778, 1.24%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.3459, 5.52%)	(0.4738, 7.57%)

* SD, %CV

Table continued from previous page
Table 27. Variance components in terms of SD and %CV – repeatability (cont'd)

Analysis variable	Sample level	Assay	# of observations	Mean	Bet. Day*	Bet. Run N*	Bet. Rgq*	Bet. Kit lot*	Bet. Operator*	Residual*	Total
Green C_T	2xLOD	12ALA	28	32.09	(0.0000, 0.00%)	(0.1314, 0.41%)	(0.0000, 0.00%)	(0.0675, 0.21%)	(0.1073, 0.33%)	(0.1158, 0.36%)	(0.1957, 0.61%
		12ARG	28	31.50	(0.0000, 0.00%)	(0.2598, 0.82%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.3324, 1.06%)	(0.4189, 1.33%
		12ASP	28	31.30	(0.0000, 0.00%)	(0.1891, 0.60%)	(0.0000, 0.00%)	(0.0920, 0.29%)	(0.0000, 0.00%)	(0.1800, 0.58%)	(0.2667, 0.85%
		12CYS	28	32.07	(0.0000, 0.00%)	(0.2523, 0.79%)	(0.0000, 0.00%)	(0.1606, 0.50%)	(0.2011, 0.63%)	(0.1512, 0.47%)	(0.3388, 1.06%
		12SER	28	32.06	(0.0000, 0.00%)	(0.2049, 0.64%)	(0.0000, 0.00%)	(0.1250, 0.39%)	(0.1177, 0.37%)	(0.1263, 0.39%)	(0.2648, 0.83%
		12VAL	28	30.65	(0.0000, 0.00%)	(0.1772, 0.58%	(0.0000, 0.00%)	(0.1198, 0.39%)	(0.1063, 0.35%)	(0.1656, 0.54%)	(0.2639, 0.86%
		13ASP	28	31.98	(0.0000, 0.00%)	(0.3773, 1.18%)	(0.0497, 0.16%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1813, 0.57%)	(0.4138, 1.29%
	LOD	12ALA	40	32.86	(0.0000, 0.00%)	(0.2332, 0.71%)	(0.0516, 0.16%)	(0.0840, 0.26%)	(0.1319, 0.40%)	(0.1780, 0.54%)	(0.3144, 0.96%)
		12ARG	40	31.90	(0.0000, 0.00%)	(0.2186, 0.69%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2289, 0.72%)	(0.1519, 0.48%)	(0.3106, 0.97%)
		12ASP	40	31.02	(0.0000, 0.00%)	(0.1762, 0.57%)	(0.1093, 0.35%)	(0.1296, 0.42%)	(0.2492, 0.80%)	(0.1005, 0.32%)	(0.2908, 0.94%)
		12CYS	40	33.14	(0.1216, 0.37%)	(0.0493, 0.15%)	(0.0000, 0.00%)	(0.3468, 1.05%)	(0.0501, 0.15%)	(0.3155, 0.95%)	(0.4216, 1.27%)
		12SER	40	33.08	(0.0832, 25%)	(0.2591, 0.78%)	(0.0000, 0.00%)	(0.2424, .73%)	(0.0000, 0.00%)	(0.2258, 0.68%)	0.3864, 1.17%
		12VAL	40	31.62	(0.2858, 0.90%)	(0.0951, 0.30%)	(0.0000, 0.00%)	(0.2244, 0.71%)	(0.0344, 0.11%)	(0.1763, 0.56%)	0.3432, 1.09%
		13ASP	38	33.09	(0.3237, 0.98%)	(0.1009, 0.31%)	(0.1409, 0.43%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2785, 0.84%)	0.4133,

* SD, %CV

Table continued from previous page
Table 27. Variance components in terms of SD and %CV – repeatability (cont'd)

Analysis variable	Sample level	Assay	# of observations	Mean	Bet. Day*	Bet. Run N*	Bet. Rgq*	Bet. Kit lot*	Bet. Operator*	Residual*	Total
Yellow C_T	2xLOD	12ALA	28	33.20	(0.0000, 0.00%)	(0.0515, 0.16%)	(0.0330, 0.10%)	(0.0000, 0.00%)	(0.0607, 0.18%)	(0.2117, 0.64%)	(0.2235, 0.67%)
		12ARG	28	33.05	(0.1397, 0.42%)	(0.1321, 0.40%)	(0.0000, 0.00%)	(0.2393, 0.72%)	(0.0000, 0.00%)	(0.3792, 1.15%)	(0.4559, 1.38%)
		12ASP	28	33.00	(0.0597, 0.18%)	(0.2131, 0.65%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1313, 0.40%)	(0.1954, 0.59%)	(0.3059, 0.93%)
		12CYS	28	33.19	(0.0646, 0.19%)	(0.0971, 0.29%)	(0.0233, 0.07%)	(0.0679, 0.20%)	(0.0863, 0.26%)	(0.1943, 0.59%)	(0.2378, 0.72%)
		12SER	28	32.85	(0.0525, 0.16%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0337, 0.10%)	(0.0937, 0.29%)	(0.1320, 0.40%)	(0.1588, 0.48%)
		12VAL	28	33.11	(0.0000, 0.00%)	(0.1026, 0.31%)	(0.0000, 0.00%)	(01469, 0.44%)	(0.1469, 0.44%)	(0.2912, 0.88%)	(0.3458, 1.04%)
		13ASP	28	33.03	(0.0000, 0.00%)	(0.1928, 0.58%)	(0.1015, 0.31%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1450, 0.44%)	(0.2493, 0.75%)
	LOD	12ALA	40	33.37	(0.0000, 0.00%)	(0.2010, 0.60%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1942, 0.58%)	(0.2177, 0.65%)	(0.3257, 0.98%)
		12ARG	40	33.14	(0.0000, 0.00%)	(0.2168, 0.65%)	(0.0000, 0.00%)	(0.3061, 0.92%)	(0.1637, 0.49%)	(0.1748, 0.53%)	(0.3717, 1.12%0
		12ASP	40	32.98	(0.0000, 0.00%)	(0.2599, 0.79%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1735, 0.53%)	(0.2228, 0.68%)	(0.3618, 1.10%)
		12CYS	40	33.31	(0.0000, 0.00%)	(0.2028, 0.61%)	(0.0000, 0.00%)	(0.1209, 0.36%)	(0.0000, 0.00%)	(0.2132, 0.64%)	(0.3050, 0.92%)
		12SER	40	33.08	(0.1254, 0.38%)	(0.2847, 0.86%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1505, 0.46%)	(0.3263, 0.99%)
		12VAL	40	33.29	(0.3133, 0.94%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2621, 0.79%)	(0.3574, 1.07%)
		13ASP	40	33.13	(0.1101, 0.33%)	(0.1326, 0.40%)	(0.1666, 0.50%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1925, 0.58%)	(0.2804, 0.85%)

* SD, %CV

Table continued from previous page
Table 27. Variance components in terms of SD and %CV – repeatability (cont'd)

Analysis variable	Sample level	Assay	# of observations	Mean	Bet. Day*	Bet. Run N*	Bet. Rgq*	Bet. Kit lot*	Bet. Operator*	Residual*	Total
Yellow C_T	WT	12ALA	28	33.41	(0.1443, 0.43%)	(0.1997, 0.60%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2269, 0.68%)	(0.3248, 0.97%)
		12ARG	28	33.30	(0.0875, 0.26%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.4098, 1.23%)	(0.0904, 0.27%)	(0.2368, 0.71%)	(0.3983, 1.20%)
		12ASP	28	33.12	(0.1591, 0.48%)	(0.1748, 0.53%)	(0.0477, 0.14%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2131, 0.64%)	(0.3075, 0.93%)
		12CYS	28	33.42	(0.0000, 0.00%)	(0.2009, 0.60%)	(0.0000, 0.00%)	(0.1444, 0.43%)	(0.0000, 0.00%)	(0.2121, 0.63%)	(0.3077, 0.92%)
		12SER	28	33.22	(0.2485, 0.75%)	(0.0000, 0.00%)	(0.0633, 0.19%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1497, 0.45%)	(0.2517, 0.76%)
		12VAL	28	33.35	(0.0000, 0.00%)	(0.2591, 0.78%)	(0.0000, 0.00%)	(0.1429, 0.43%)	(0.0000, 0.00%)	(0.2721, 0.82%)	(0.3863, 1.16%)
		13ASP	28	33.45	(0.0000, 0.00%)	(0.1194, 0.36%)	(0.0526, 0.16%)	(0.0341, 0.10%)	(0.0000, 0.00%)	(0.1651, 0.49%)	(0.2078, 0.62%)

* SD, %CV

The precision of the *therascreen* KRAS RGQ PCR Kit between-laboratories (reproducibility) was assessed. Three different laboratories (test sites) were used. The same test panel was used for this study as for the repeatability study. At each site, laboratory conditions were varied by RGQ instrument, operator, KRAS Kit lot, and runs per day to yield a total of 88 runs per site over 22 non-consecutive days.

The proportion of correct mutation calls is reported in Table 28. The quantitative precision values are presented in Table 29. The total reproducibility of the KRAS kit is shown in the #Total (SD, CV%) column of Table 29.

Table 27. Proportion of correct mutation calls across all sites

Grouping variables		Proportion		Two-sided 95% confidence limit	
Sample level	Assay	Fraction	Percentage	Lower	Upper
2xLOD	12ALA	84/84	100.00%	95.70%	100.00%
	12ARG*	84/84	100.00%	95.70%	100.00%
	12ASP	84/84	100.00%	95.70%	100.00%
	12CYS	84/84	100.00%	95.70%	100.00%
	12SER*	84/84	100.00%	95.70%	100.00%
	12VAL	84/84	100.00%	95.70%	100.00%
	13ASP*	84/84	100.00%	95.70%	100.00%
LOD	12ALA	118/120	98.33%	94.11%	99.80%
	12ARG	120/120	100.00%	96.97%	100.00%
	12ASP	120/120	100.00%	96.97%	100.00%
	12CYS	119/120	99.17%	95.44%	99.98%
	12SER*	120/120	100.00%	96.97%	100.00%
	12VAL	120/120	100.00%	96.97%	100.00%
	13ASP*	118/120	98.33%	94.11%	99.80%
WT	All	82/84	97.62%	91.66%	99.71%

* Represented by FFPE cell line

Table 28. Variance components in terms of SD and %CV – reproducibility

Analysis variable	Sample level	Assay	# of observations	Mean	Between site*	Between day within site*	Between Run N within site*	Between Rgq within site*	Bet. Kit lot within site*	Bet. Operator within site*	Residual*	Total
Delta C$_t$	2xLOD	12ALA	84	5.48	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1669, 3.05%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1287, 2.35%)	(0.1679, 3.07%)	(0.2640, 4.82%)
		12ARG	84	4.81	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1172, 2.43%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2729, 5.67%)	(0.2967, 6.16%)
		12ASP	84	4.57	(0.0000, 0.00%)	(0.0943, 2.06%)	(0.1457, 3.19%)	(0.0000, 0.00%)	(0.0600, 1.31%)	(0.1718, 3.76%)	(0.1565, 3.43%)	(0.2854, 6.25%)
		12CYS	84	5.61	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2060, 3.67%)	(0.0264, 0.47%)	(0.0698, 1.24%)	(0.0000, 0.00%)	(0.1671, 2.98%)	(0.2728, 4.87%)
		12SER	84	5.34	(0.0000, 0.00%)	(0.1362, 2.55%)	(0.1669, 3.13%)	(0.1527, 2.86%)	(0.0000, 0.00%)	(0.2020, 3.79%)	(0.2382, 4.46%)	(0.3902, 7.31%)
		12VAL	84	4.13	(0.0874, 2.11%)	(0.0000, 0.00%)	(0.1677, 4.06%)	(0.0000, 0.00%)	(0.0869, 2.10%)	(0.0000, 0.00%)	(0.2711, 6.56%)	(0.3359, 8.12%)
		13ASP	84	5.22	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2161, 4.14%)	(0.2712, 5.20%)	(0.0000, 0.00%)	(0.1930, 3.70%)	(0.2275, 4.36%)	(0.4279, 8.20%)
	LOD	12ALA	119	6.33	(0.0000, 0.00%)	(0.0410, 0.65%)	(0.1207, 1.91%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0247, 0.39%)	(0.2640, 4.17%)	(0.2936, 4.64%)
		12ARG	120	5.42	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1797, 3.31%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1872, 3.45%)	(0.2590, 4.78%)
		12ASP	120	4.66	(0.1183, 2.54%)	(0.0646, 1.38%)	(0.2121, 4.55%)	(0.0261, 0.56%)	(0.0217, 0.46%)	(0.0440, 0.94%)	(0.1455, 3.12%)	(0.2862, 6.14%)
		12CYS	120	6.54	(0.0000, 0.00%)	(0.0132, 0.20%)	(0.1775, 2.72%)	(0.0000, 0.00%)	(0.1621, 2.48%)	(0.1708, 2.61%)	(0.4202, 6.43%)	(0.4981, 7.62%)
		12SER	120	6.28	(0.0000, 0.00%)	(0.0824, 1.31%)	(0.2271, 3.62%)	(0.0775, 1.24%)	(0.0000, 0.00%)	(0.2383, 3.80%)	(0.3164, 5.04%)	(0.4570, 7.28%)
		12VAL	120	5.05	(0.0315, 0.62%)	(0.1648, 3.26%)	(0.0955, 1.89%)	(0.0703, 1.39%)	(0.0320, 0.63%)	(0.0795, 1.57%)	(0.2120, 4.20%)	(0.2965, 5.87%)
		13ASP	118	6.17	(0.0000, 0.00%)	(0.1673, 2.71%)	(0.1987, 3.22%)	(0.2332, 3.78%)	(0.0000, 0.00%)	(0.0843, 1.37%)	(0.3075, 4.99%)	(0.4488, 7.28%)

** SD, %CV

Table continued from previous page
Table 29. Variance components in terms of SD and %CV – reproducibility (cont'd)

Analysis variable	Sample level	Assay	# of observations	Mean	Between site*	Between day within site*	Bet. Run N within site*	Bet. Rgq within site*	Bet. Kit lot within site*	Bet. Operator within site*	Residual*	Total
Green C$_t$	2xLOD	12ALA	84	32.13	(0.1578, 0.49%)	(0.0000, 0.00%)	(0.2509, 0.78%)	(0.0745, 0.23%)	(0.0000, 0.00%)	(0.1249, 0.39%)	(0.1362, 0.42%)	(0.3390, 1.06%)
		12ARG	84	31.61	(0.0882, 0.28%)	(0.0000, 0.00%)	(0.2430, 0.77%)	(0.1339, 0.42%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2604, 0.82%)	(0.3828, 1.21%)
		12ASP	84	31.24	(0.1655, 0.53%)	(0.0391, 0.13%)	(0.2178, 0.70%)	(0.0600, 0.19%)	(0.0000, 0.00%)	(0.2052, 0.66%)	(0.1426, 0.46%)	(0.3542, 1.13%)
		12CYS	84	32.15	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2836, 0.88%)	(0.0852, 0.26%)	(0.0940, 0.29%)	(0.1658, 0.52%)	(0.1318, 0.41%)	(0.3636, 1.13%)
		12SER	84	32.14	(0.1457, 0.45%)	(0.0000, 0.00%)	(0.2659, 0.83%)	(0.1807, 0.56%)	(0.0000, 0.00%)	(0.2715, 0.84%)	(0.1783, 0.55%)	(0.4554, 1.42%)
		12VAL	84	30.69	(0.0646, 0.21%)	(0.0480, 0.16%)	(0.2124, 0.69%)	(0.0000, 0.00%)	(0.1031, 0.34%)	(0.0000, 0.00%)	(0.2000, 0.65%)	(0.3143, 1.02%)
		13ASP	84	32.12	(0.2111, 0.66%)	(0.0000, 0.00%)	(0.3218, 1.00%)	(0.2966, 0.92%)	(0.0000, 0.00%)	(0.1743, 0.54%)	(0.1980, 0.62%)	(0.5184, 1.61%)
	LOD	12ALA	119	32.93	(0.0000, 0.00%)	(0.1524, 0.46%)	(0.1821, 0.55%)	(0.1048, 0.32%)	(0.0757, 0.23%)	(0.1007, 0.31%)	(0.2526, 0.77%)	(0.3721, 1.13%)
		12ARG	120	31.98	(0.0000, 0.00%)	(0.0743, 0.23%)	(0.1936, 0.61%)	(0.1262, 0.39%)	(0.0000, 0.00%)	(0.1332, 0.42%)	(0.1619, 0.51%)	(0.3096, 0.97%)
		12ASP	120	31.06	(0.1880, 0.61%)	(0.1184, 0.38%)	(0.1681, 0.54%)	(0.1033, 0.33%)	(0.1171, 0.38%)	(0.1481, 0.48%)	(0.1333, 0.43%)	(0.3511, 1.13%)
		12CYS	120	33.19	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2513, 0.76%)	(0.0776, 0.23%)	(0.2128, 0.64%)	(0.1427, 0.43%)	(0.2712, 0.82%)	(0.4401, 1.33%)
		12SER	120	33.13	(0.2194, 0.66%)	(0.0000, 0.00%)	(0.2433, 0.73%)	(0.1263, 0.38%)	(0.1470, 0.44%)	(0.1973, 0.60%)	(0.2052, 0.62%)	(0.4437, 1.34%)
		12VAL	120	31.65	(0.0000, 0.00%)	(0.1254, 0.40%)	(0.1645, 0.52%)	(0.1307, 0.41%)	(0.1271, 0.40%)	(0.0976, 0.31%)	(0.1792, 0.57%)	(0.3159, 1.00%)
		13ASP	118	33.08	(0.0000, 0.00%)	(0.1789, 0.54%)	(0.1661, 0.50%)	(0.3569, 1.08%)	(0.0649, 0.20%)	(0.1565, 0.47%)	(0.2588, 0.78%)	(0.4894, 1.48%)

** SD, %CV

Table continued from previous page
Table 29. Variance components in terms of SD and %CV – reproducibility (cont'd)

Analysis variable	Sample level	Assay	# of observations	Mean	Between site*	Between day within site*	Bet. Run N within site*	Bet. Rgq within site*	Bet. Kit lot within site*	Bet. Operator within site*	Residual*	Total
Yellow C$_t$	2xLOD	12ALA	84	**33.25**	(0.0706, 0.21%)	(0.0399, 0.12%)	(0.1314, 0.40%)	(0.1303, 0.39%)	(0.0000, 0.00%)	(0.1124, 0.34%)	(0.1913, 0.58%)	(0.2883, 0.87%)
		12ARG	84	**33.07**	(0.0000, 0.00%)	(0.1406, 0.43%)	(0.1353, 0.41%)	(0.0000, 0.00%)	(0.2024, 0.61%)	(0.1262, 0.38%)	(0.2831, 0.86%)	(0.4016, 1.21%)
		12ASP	84	**32.98**	(0.0000, 0.00%)	(0.0480, 0.15%)	(0.1706, 0.52%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0797, 0.24%)	(0.1795, 0.54%)	(0.2616, 0.79%)
		12CYS	84	**33.20**	(0.0000, 0.00%)	(0.0976, 0.29%)	(0.1781, 0.54%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1454, 0.44%)	(0.1723, 0.52%)	(0.2939, 0.89%)
		12SER	84	**32.91**	(0.0000, 0.00%)	(0.1101, 0.33%)	(0.0549, 0.17%)	(0.0669, 0.20%)	(0.0677, 0.21%)	(0.1186, 0.36%)	(0.2274, 0.69%)	(0.2916, 0.89%)
		12VAL	84	**33.17**	(0.0688, 0.21%)	(0.0000, 0.00%)	(0.1896, 0.57%)	(0.0937, 0.28%)	(0.1140, 0.34%)	(0.1311, 0.40%)	(0.2605, 0.79%)	(0.3768, 1.14%)
		13ASP	84	**33.10**	(0.0000, 0.00%)	(0.0482, 0.15%)	(0.2035, 0.61%)	(0.0000, 0.00%)	(0.0466, 0.14%)	(0.1460, 0.44%)	(0.1688, 0.51%)	(0.3019, 0.91%)
	LOD	12ALA	119	**33.33**	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2108, 0.63%)	(0.0820, 0.25%)	(0.0000, 0.00%)	(0.1443, 0.43%)	(0.2253, 0.68%)	(0.3411, 1.02%)
		12ARG	120	**33.15**	(0.1092, 0.33%)	(0.0537, 0.16%)	(0.1605, 0.48%)	(0.0507, 0.15%)	(0.2157, 0.65%)	(0.1276, 0.39%)	(0.2180, 0.66%)	(0.3749, 1.13%)
		12ASP	120	**32.96**	(0.0000, 0.00%)	(0.0832, 0.25%)	(0.2022, 0.61%)	(0.0864, 0.26%)	(0.0000, 0.00%)	(0.1117, 0.34%)	(0.2223, 0.67%)	(0.3343, 1.01%)
		12CYS	120	**33.26**	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2232, 0.67%)	(0.1691, 0.51%)	(0.0789, 0.24%)	(0.0000, 0.00%)	(0.2097, 0.63%)	(0.3516, 1.06%)
		12SER	120	**33.01**	(0.1573, 0.48%)	(0.0716, 0.22%)	(0.2134, 0.65%)	(0.0951, 0.29%)	(0.0000, 0.00%)	(0.0784, 0.24%)	(0.1689, 0.51%)	(0.3263, 0.99%)
		12VAL	120	**33.25**	(0.1519, 0.46%)	(0.1960, 0.59%)	(0.1272, 0.38%)	(0.0000, 0.00%)	(0.1298, 0.39%)	(0.0553, 0.17%)	(0.2162, 0.65%)	(0.3487, 1.05%)
		13ASP	118	**33.16**	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1768, 0.53%)	(0.0998, 0.30%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1973, 0.59%)	(0.2802, 0.84%)

** SD, %CV

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Table 29. Variance components in terms of SD and %CV – reproducibility (cont'd)

Analysis variable	Sample level	Assay	# of observations	Mean	Between site*	Between day within site*	Bet. Run N within site*	Bet. Rgq within site*	Bet. Kit lot within site*	Bet. Operator within site*	Residual*	Total
	WT	12ALA	84	33.44	(0.1257, 0.38%)	(0.0961, 0.29%)	(0.1845, 0.55%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.2083, 0.62%)	(0.3104, 0.93%)
		12ARG	84	33.37	(0.1191, 0.36%)	(0.0000, 0.00%)	(0.1869, 0.56%)	(0.1321, 0.40%)	(0.2529, 0.76%)	(0.1205, 0.36%)	(0.2132, 0.64%)	(0.4217, 1.26%)
		12ASP	84	33.16	(0.0574, 0.17%)	(0.0738, 0.22%)	(0.2162, 0.65%)	(0.0563, 0.17%)	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1844, 0.56%)	(0.2997, 0.90%)
		12CYS	84	33.42	(0.0000, 0.00%)	(0.0000, 0.00%)	(0.1964, 0.59%)	(0.0720, 0.22%)	(0.1311, 0.39%)	(0.0262, 0.08%)	(0.2258, 0.68%)	(0.3287, 0.98%)
		12SER	84	33.20	(0.0812, 0.24%)	(0.1331, 0.40%)	(0.1734, 0.52%)	(0.0329, 0.10%)	(0.0000, 0.00%)	(0.2009, 0.61%)	(0.1923, 0.58%)	(0.3535, 1.06%)
		12VAL	84	33.41	(0.0000, 0.00%)	(0.0695, 0.21%)	(0.2046, 0.61%)	(0.0000, 0.00%)	(0.1708, 0.51%)	(0.1437, 0.43%)	(0.2339, 0.70%)	(0.3799, 1.14%)
		13ASP	84	33.46	(0.0000, 0.00%)	(0.0613, 0.18%)	(0.1802, 0.54%)	(0.0744, 0.22%)	(0.0073, 0.02%)	(0.0969, 0.29%)	(0.1790, 0.53%)	(0.2816, 0.84%)

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 µm 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 DCT 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.

An additional Sample Handling study was conducted using clinical FFPE clinical NSCLC samples representative of 12ALA, 12ARG, 12SER, and 13ASP mutations, as the previous study used cell line samples representative of these mutations. This additional study followed the same study design as the previous study. All sample preparations for 12ALA, 12ARG, and 13ASP mutant samples extracted across all three individual test sites, provided a correct mutation call when tested with the KRAS Kit. The overall correct call for these samples was 100%. Sample preparations for 12SER mutation provided a frequency of correct mutation call

of 28/30 (percentage of correct call equal to 93.33%) across all three individual test sites. The results demonstrate the consistency of the DNA extraction procedure and sample processing workflow used to perform testing with 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. Each sample was extracted and tested with the *therascreen* KRAS RGQ PCR Kit.

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.

The primary analysis was based on the specific mutation being detected across acquisition types. The measures of overall percentage agreement, positive percentage agreement, and negative percentage agreement were calculated along with the exact two-sided 95% confidence limit for each pair-wise comparison.

Table 29. Agreement between Sample Acquisitions Methods

Comparison	Agreement	Frequencies	Percentage (%)	Lower two-sided Confidence Limit	Upper two-sided 95% Confidence Limits
CNB Vs FNA with CNB as Reference	Overall Percent Agreement	148/156	94.87	90.15	97.76
	Positive Percent Agreement	29/35	82.86	66.35	93.44
	Negative Percent Agreement	119/121	98.35	94.16	99.80
CNB Vs RES with CNB as Reference	Overall Percent Agreement	153/161	95.03	90.44	97.83
	Positive Percent Agreement	31/37	83.78	67.99	93.81
	Negative Percent Agreement	122/124	98.39	94.30	99.80
FNA Vs CNB with FNA as Reference	Overall Percent Agreement	148/156	94.87	90.15	97.76
	Positive Percent Agreement	29/32	90.63	74.98	98.02
	Negative Percent Agreement	119/124	95.97	90.84	98.68
FNA Vs RES with FNA as Reference	Overall Percent Agreement	152/156	97.44	93.57	99.30
	Positive Percent Agreement	30/32	93.75	79.19	99.23
	Negative Percent Agreement	122/124	98.39	94.30	99.80

Table 30. Agreement between Sample Acquisitions Methods (con't)

Comparison	Agreement	Frequencies	Percentage (%)	Lower two-sided Confidence Limit	Upper two-sided 95% Confidence Limits
RES Vs. CNB with RES as Reference	Overall Percent Agreement	153/161	95.03	90.44	97.83
	Positive Percent Agreement	31/34	91.18	76.32	98.14
	Negative Percent Agreement	122/127	96.06	91.05	98.71
RES Vs. FNA with RES as Reference	Overall Percent Agreement	152/156	97.44	93.57	99.30
	Positive Percent Agreement	30/32	93.75	79.19	99.23
	Negative Percent Agreement	122/124	98.39	94.30	99.80

In addition, Passing-Bablok and Deming regression analysis was performed to compare C_T and ΔC_T values between different sample acquisition methods. The regression analysis demonstrated that there is no evidence to suggest that there exists any constant or proportional difference between the RES, CNB, and FNA sample types in terms of C_T or ΔC_T. A linear regression analysis was also performed to investigate the effect of both percentage of necrotic and tumour tissue on the relevant ΔC_T values. The slope of the regression line for both necrotic and percent tumour vs ΔC_T indicates that there is no evidence to suggest that there is any meaningful difference in ΔC_T values with increasing values of the percentage or necrotic or tumor tissue.

Clinical Performance

The *therascreen* KRAS RGQ PCR Kit is designed to specifically detect 7 *KRAS* mutations in codons 12 and 13 of the *KRAS* gene. It is not designed to specifically detect the wild-type sequence at these codons. The results of the test are reported out as "[mutation name] Detected" and "No Mutation Detected." In clinical studies presented below, *KRAS* mutation-positive results are those patients whose tumor tissue was positive for one or more of the 7 mutations detected by the *therascreen* KRAS RGQ PCR Kit (G12A, G12D, G12R, G12C, G12S, G12V, G13D). *KRAS* mutation-negative (wild-type) refers to those patients whose tumor tissue was negative for the 7 mutations detected by the *therascreen* KRAS RGQ PCR Kit (i.e., the sample may, in fact, harbor mutations in the *KRAS* gene not identified by the *therascreen* KRAS RGQ PCR Kit).

Clinical study supporting use with Erbitux (cetuximab)

A clinical performance study was conducted to generate data to support the clinical utility of the *therascreen* KRAS RGQ PCR Kit as a diagnostic tool that enables selection of patients for treatment with Erbitux (cetuximab). The safety and effectiveness of Erbitux (cetuximab) has been demonstrated in the CA225025 study. CA225025 was a multicenter, open-label, randomized clinical trial conducted in 572 patients with EGFR-expressing, previously treated, recurrent metastatic CRC (mCRC). Patients were randomized (1:1) to receive either Erbitux (cetuximab) plus best supportive care (BSC) or BSC alone. Erbitux (cetuximab) was administered as a 400 mg/m^2 initial dose, followed by 250 mg/m^2 weekly until disease progression or unacceptable toxicity.

Of the 572 randomized patients, the median age was 63 years; 64% were male, 89% were Caucasian and 77% had baseline ECOG performance status of 0–1. Demographics and baseline characteristics were similar between study arms. All patients were to have received and progressed on prior therapy including an irinotecan-containing regimen and an oxaliplatin-containing regimen.

KRAS mutation status was available for 453/572 (79%) of the patients: 245 (54%) patients had *KRAS* mutation-negative tumors and 208 (46%) patients had *KRAS* mutation-positive tumors when assessed by the *therascreen* KRAS RGQ PCR Kit.

The main outcome measure of the study was overall survival (OS). For the KRAS mutation-negative (wild-type) population, median survival time (95% CI) was 8.6 (7.0, 10.3) months in the Erbitux (cetuximab)+BSC group and 5.0 (4.3, 5.7) months in the BSC group. The OS hazard ratio of Erbitux (cetuximab)+BSC over BSC was 0.63. The 95% confidence interval (CI) was (0.47, 0.84).

For the KRAS mutation-positive population, median survival time was 4.8 (3.9, 5.6) months in the Erbitux (cetuximab)+BSC group and 4.6 (3.6, 4.9) months in the BSC group. The hazard ratio was 0.91 with 95% CI (0.67, 1.24). Results are presented in Table 31 and Figure 25.

Table 30. Overall survival in previously treated EGFR-expressing metastatic colorectal cancer (all randomized and KRAS status)

	All randomized		Wild-type *KRAS* mutation-negative		*KRAS* mutation-positive	
	Erbitux+BSC N=287	BSC N=285	Erbitux+BSC N=117	BSC N=128	Erbitux+BSC N=108	BSC N=100
Median (months) (95% CI)	6.1 (5.4, 6.7)	4.6 (4.2, 4.9)	8.6 (7.0, 10.3)	5.0 (4.3, 5.7)	4.8 (3.9, 5.6)	4.6 (3.6, 4.9)
Hazard ratio (95% CI)	0.77 (0.64, 0.92)		0.63 (0.47, 0.84)		0.91 (0.67, 1.24)	
p-value*	0.0046		–		–	

* Based on the stratified log-rank test.
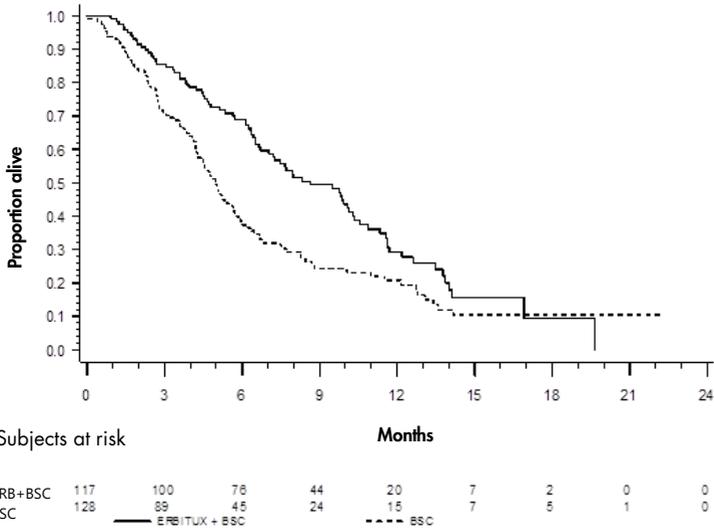
Figure 23. Kaplan-Meier curve for overall survival in patients with KRAS mutation-negative (wild-type) metastatic colorectal cancer.

Overall survival rates based on Kaplan-Meier estimates at months 6 and 12 were higher for the Erbitux (cetuximab)+BSC group than the BSC group for the *KRAS* wild-type subset. This advantage was not observed in the *KRAS* mutant subset.

Clinical study supporting use with Vectibix (panitumumab)

A clinical performance study was conducted to generate data to support the clinical utility of the *therascreen* KRAS RGQ PCR Kit as a diagnostic tool that aids in the identification of patients for treatment with Vectibix (panitumumab). The objective of the study was to assess whether *KRAS* mutation status as determined by the *therascreen* KRAS RGQ PCR Kit can be used to select patients with mCRC who will benefit from Vectibix (panitumumab) treatment. The clinical trial 20050203 was a multicenter, prospective, open-label, randomized phase 3 study to assess the efficacy of panitumumab in combination with oxaliplatin, 5-fluorouracil (5-FU) and leucovorin (FOLFOX) vs. FOLFOX alone in patients with previously untreated, recurrent mCRC.

Banked tumor samples from patients in study 20050203 were tested with the *therascreen* KRAS RGQ PCR Kit to identify two subgroups: *KRAS* mutation-positive (mutant *KRAS*) and *KRAS* mutation-negative (wild-type *KRAS*), according to whether at least one or none of 7 *KRAS* mutations in codons 12 and 13 of exon 2 in the *KRAS* gene was detected. In retrospective analyses, efficacy data from study 20050203 were stratified by *KRAS* subgroup. The primary objective of the *KRAS* analysis was to assess whether an overall improvement in PFS for Vectibix (panitumumab) plus FOLFOX relative to FOLFOX alone was significantly greater among subjects with *KRAS* wild-type tumors compared to subjects with *KRAS* mutant tumors.

The prespecified primary efficacy endpoint was progression-free survival (PFS) in the group of patients (n = 656) with wild-type *KRAS* mCRC as assessed by a blinded independent central review of imaging. Other key efficacy endpoints included OS and ORR. The efficacy results in patients with wild-type *KRAS* mCRC are presented in Table 32 and Figure 26.

Table 31. Efficacy results in patients with wild-type KRAS mCRC

	Wild-type *KRAS* population	PFS			ORR (95% CI)
		Median (months) (95% CI)	Hazard ratio (95% CI)	p-value	
Panitumumab plus FOLFOX	N=325	9.6 (9.2, 11.1)	0.80 (0.66, 0.97)	0.02	54% (48%, 59%)
FOLFOX alone	N=331	8.0 (7.5, 9.3)			47% (41%, 52%)
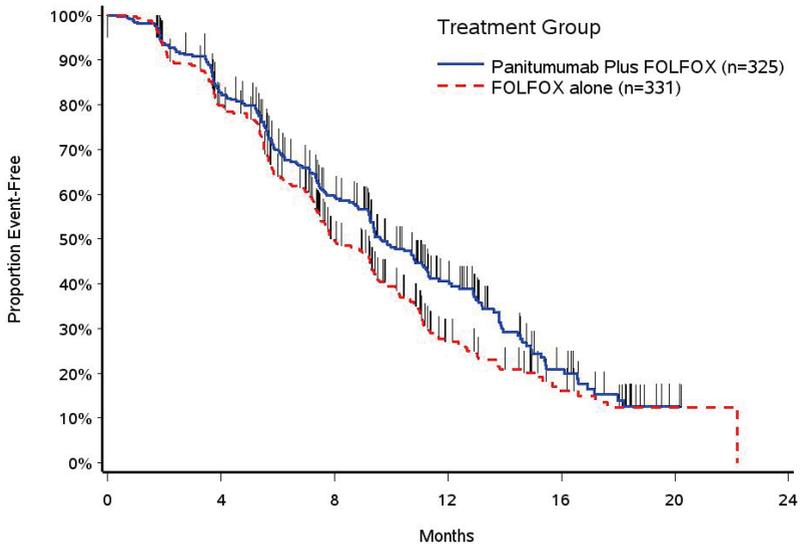
Figure 24. Kaplan-Meier plot of progression-free survival (PFS) in patients with wild-type KRAS mCRC.

Among patients with *KRAS* mutant tumors, median PFS was 7.3 months (95% CI: 6.3, 8.0) in the 221 patients receiving Vectibix (panitumumab) plus FOLFOX versus the median PFS of 8.8 months (95% CI: 7.7, 9.4) in the 219 patients who received FOLFOX alone (HR = 1.29, 95% CI: 1.04, 1.62). Median OS was 15.5 months (95% CI: 13.1, 17.6) in patients receiving Vectibix (panitumumab) plus FOLFOX versus the median OS of 19.3 months (95% CI: 16.5, 21.8) in patients who received FOLFOX alone (HR = 1.24, 95% CI: 0.98, 1.57).

An exploratory analysis of OS with updated information based on events in 82% of patients with wild-type *KRAS* mCRC estimated the treatment effect of Vectibix (panitumumab) plus FOLFOX compared with FOLFOX alone. Median OS among 325 patients with wild-type *KRAS* mCRC who received Vectibix (panitumumab) plus FOLFOX was 23.8 months (95% CI: 20.0, 27.7) versus 19.4 months (95% CI: 17.4, 22.6) among 331 patients who received FOLFOX alone (HR = 0.83, 95% CI: 0.70, 0.98). Results are shown in Figure 27.
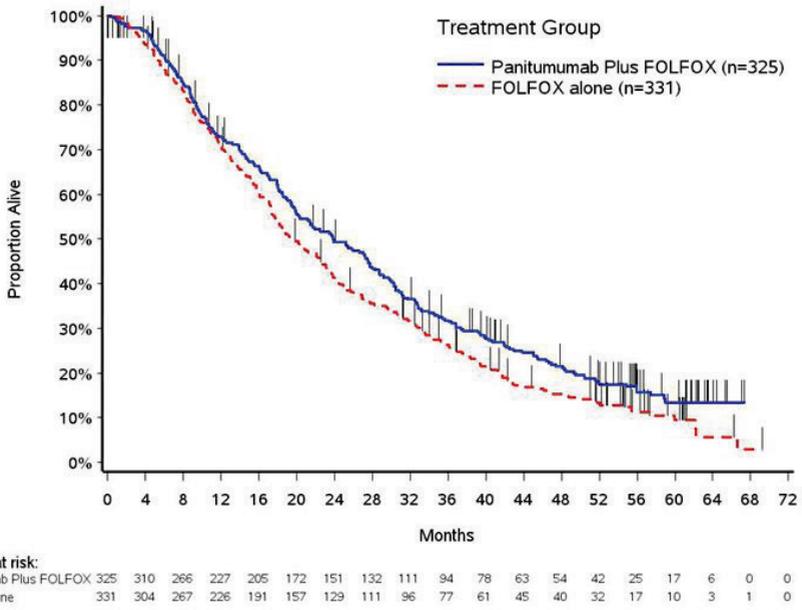
Figure 25. Kaplan-Meier Plot of overall survival (OS) in patients with Wild-type KRAS mCRC.

Clinical study supporting use of LUMYKRAS® (sotorasib)

A clinical performance study was conducted to demonstrate the clinical validity of the *therascreen* KRAS RGQ PCR Kit as a diagnostic tool to aid in the identification of *KRAS* G12C positive NSCLC patient for treatment with LUMYKRAS (sotorasib). The objective of the study was to assess whether G12C mutation status, as determined by the *therascreen* KRAS RGQ PCR Kit, can be used to select patients with advanced NSCLC who will benefit from LUMYKRAS (sotorasib) treatment. The clinical trial 20170543 was an open-label, multicenter, phase 1/2 study designed to evaluate the efficacy and safety of LUMYKRAS (sotorasib) in adult subjects with advanced solid tumors that harbor the *KRAS* G12C mutation.

Data from the primary analysis of the NSCLC phase 2 portion of this study has been used to support the clinical validity of the *therascreen* KRAS RGQ PCR Kit as a diagnostic tool.

Enrollment was restricted to subjects with *KRAS* G12C-mutated NSCLC as assessed by a local laboratory result, which was confirmed by central testing using the *therascreen* KRAS RGQ PCR Kit. The primary end point of the NSCLC phase 2 portion of this study was to evaluate tumor objective response rate (ORR) assessed by Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, criteria of LUMYKRAS (sotorasib) as monotherapy in subjects with *KRAS* G12C-mutated advanced tumors.

Of a total of 126 subjects with NSCLC, 124 subjects were included in the full analysis set. Two subjects were excluded as they did not have ≥1 measurable lesion according to blinded independent centralized review (BICR).

The primary endpoint of ORR (complete response + partial response) measured by computed tomography or magnetic resonance imaging and assessed per RECIST 1.1 by BICR laboratory for subjects with *KRAS* G12C-mutated NSCLC was 37.1% (46 of 124 subjects; 95% CI:28.6-

46.2%); three (3) subjects (2.4%) achieved complete response and 43 subjects 34.7%) achieved partial response*.

* Based on 01 December 2020 data cut.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support** (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 Specimen Collection, Preparation for Analysis, and Storage, page 17.	Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit, if necessary.
b) The *therascreen* 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 *therascreen* KRAS RGQ PCR 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 33 lists the possible flags that may be generated by the *therascreen* KRAS Assay Package, their meaning, and actions to be taken. Flags are relevant for both NSCLC and CRC.

Table 32. Rotor-Gene Q therascreen KRAS Assay Package flags meanings and recommended actions

Flag	Meaning	Action
PC_CTRL_ASSAY_FAIL	PCR run invalid — FAM C_T out of range for positive control in control reaction	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).

Table continued from previous page

Table 33. Rotor-Gene Q therascreen KRAS Assay Package flags meanings and recommended actions

Flag	Meaning	Action
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	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section (≥4 sections). Setup 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.

Flags specific for NSCLC

Table 34 lists the possible flags that may be generated by the Rotor-Gene Q *therascreen* KRAS Assay Package, the meaning of the flags and actions to be taken for NSCLC samples.

Table 33. Rotor-Gene Q therascreen KRAS Assay Package flags meanings and recommended actions for NSCLC samples

Flag	Meaning	Action
SAMPLE_INT_CTRL_ FAIL	C_T too high (or no C_T) for internal control (HEX), FAM channel mutation-negative	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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_ EARLY_CT	Mutation tube invalid — C_T HEX too low for sample (internal control)	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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.

Table 34. Rotor-Gene Q therascreen KRAS Assay Package flags meanings and recommended actions for NSCLC samples

Flag	Meaning	Action
SAMPLE_INVALID_ DATA	Mutation tube invalid — Fluorescence data in internal control can't be interpreted	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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.	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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)	Setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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.

Flags specific for CRC

The table below lists the possible flags that may be generated by the Rotor-Gene Q *therascreen* KRAS Assay Package, the meaning of the flags and actions to be taken for CRC samples.

Table 34. Rotor-Gene Q therascreen KRAS Assay Package flags meanings and recommended actions for CRC samples

Flag	Meaning	Action
SAMPLE_INT_CTRL_ FAIL	C_T too high (or no C_T) for internal control (HEX), FAM channel mutation-negative	If sample given valid status — No action. If sample given invalid status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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_ EARLY_CT	Mutation tube invalid — C_T HEX too low for sample (internal control)	If sample given valid status — No action. If sample given invalid status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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.

Table 35. Rotor-Gene Q therascreen KRAS Assay Package flags meanings and recommended actions for CRC samples (cont'd)

Flag	Meaning	Action
SAMPLE_INVALID_DATA	Mutation tube invalid — Fluorescence data in internal control can't be interpreted	If sample given valid status — No action. If sample given invalid status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup 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.

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Description
$C\epsilon$	Marking for European community
∑ \<N\>	Contains reagents sufficient for \<N\> reactions
(hourglass)	Use by
IVD	In vitro diagnostic medical device
REF	Catalog number
LOT	Lot number
MAT	Material number
CONT	Contains
NUM	Number
Rn	R is for revision of the Handbook and n is the revision number
(thermometer)	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 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 36.

Table 35. 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 5plex HRM instrument. Select the Advanced tab in the New Run window that appears.

2. To create a new template, select **Empty Run**, 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 28).
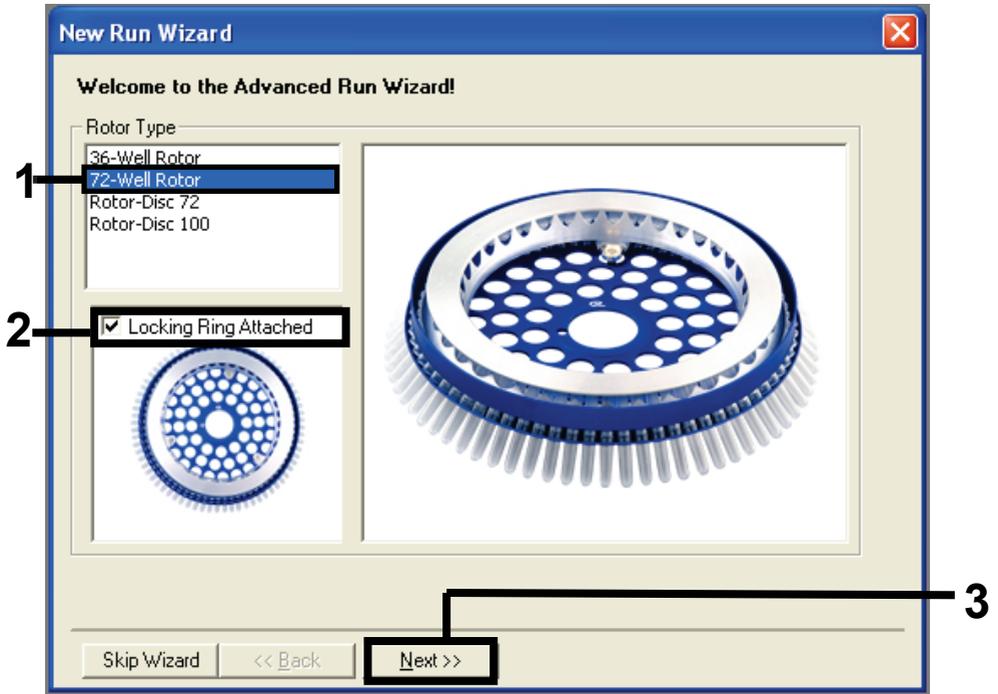
Figure 26. The New Run Wizard dialog box. 1 = Rotor type, 2 = Locking Ring Attached box, 3 =Next.

4. Enter the name of the operator. Add any notes and enter the reaction volume as **25**. Ensure that the **Sample Layout** field contains the value **1, 2, 3…**. Click **Next** (Figure 29).
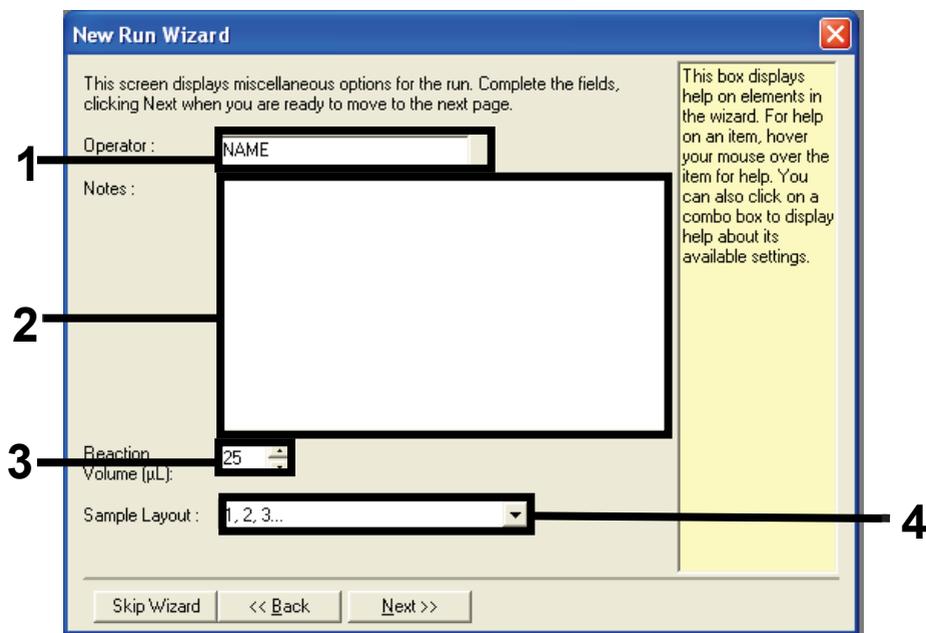
Figure 27. Entering operator name and reaction volumes. 1=Operator dialog field, 2 = Notes dialog field, 3 = Reaction Volume field, 4 = Sample Layout, 5 = Next.

5. Click **Edit Profile** in the New Run Wizard window (Figure 30) and program the temperature profile according to the information in the following steps.
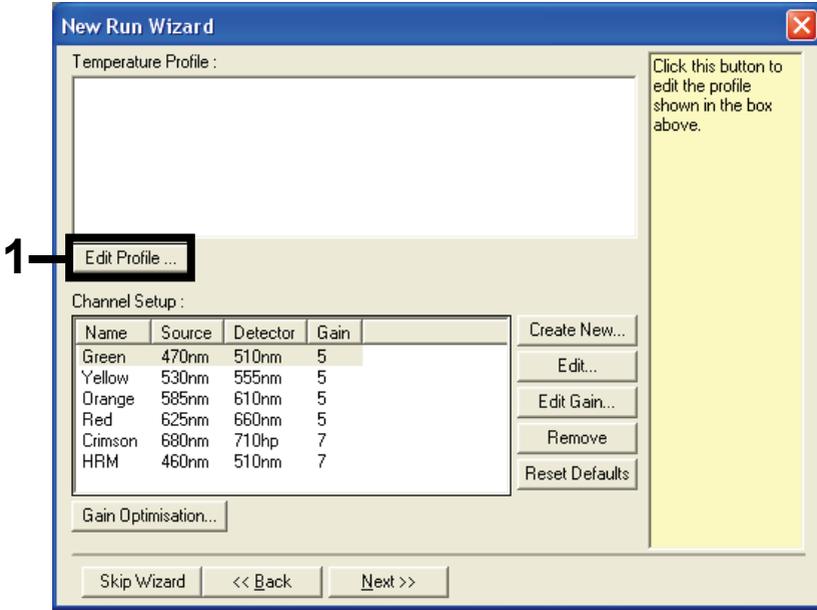
Figure 28. Editing the profile.

6. Click **Insert after** and select **New Hold at Temperature** (Figure 31).
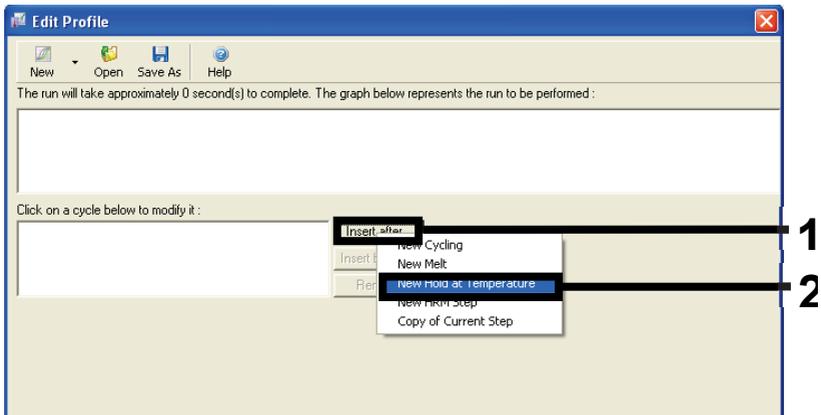
Figure 29. Inserting an initial incubation step. 1 = Insert after, 2 = New Hold at Temperature.

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7. Set the value in the **Hold Temperature** field to **95°C** and the **Hold Time** field to **15 mins 0 secs**. Click **Insert After**, then select **New Cycling** (Figure 32).
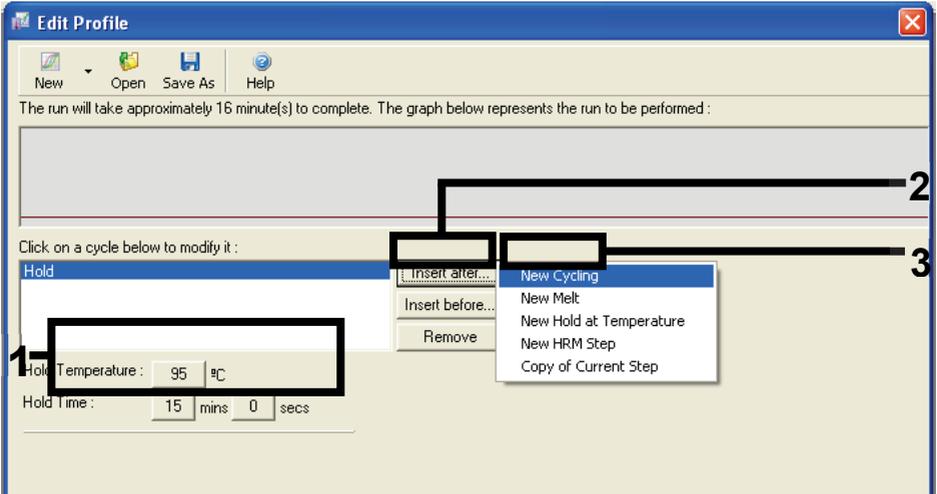
Figure 30. Initial incubation step at 95°C. 1 = Hold Temperature and Hold Time, 2 = Insert after, 3 = New Cycling.

8. Set the number of cycle repeats to **40**. Select the first step and set to **95°C for 30 secs** (Figure 33).
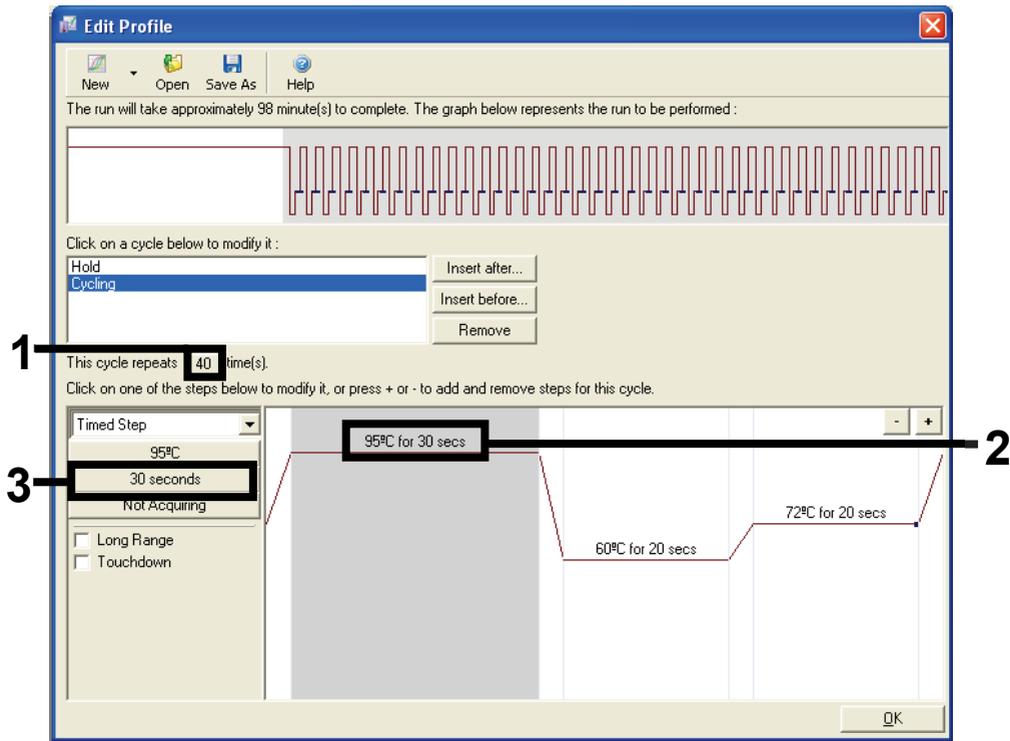
Figure 31. Cycling step at 95°C. 1 = Cycle repeats box, 2 = step one temperature setting, 3 = step one time setting.

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9. Highlight the second step and set to **60°C for 60 secs**. To enable data acquisition during this step, click **Not Acquiring** (Figure 34).
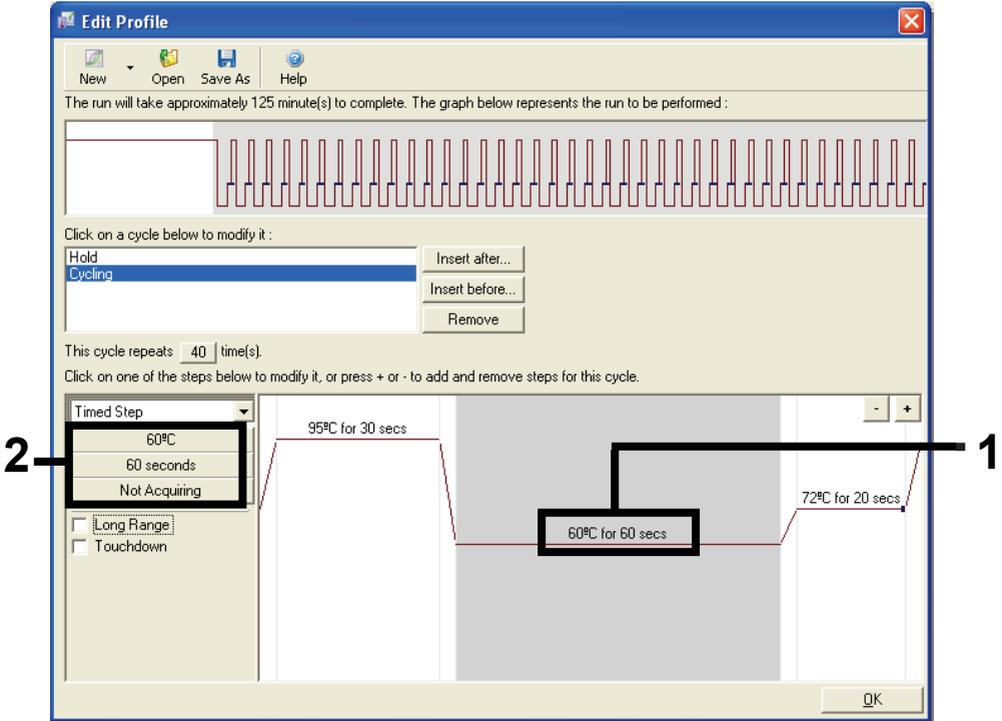
Figure 32. Cycling step at 60°C. 1 = second step temperature and time setting, 2 = Not Acquiring.

10. In the Available Channels list, select **Green** and **Yellow** then click **>** to move them to Acquiring Channels list. Click **OK** (Figure 35).
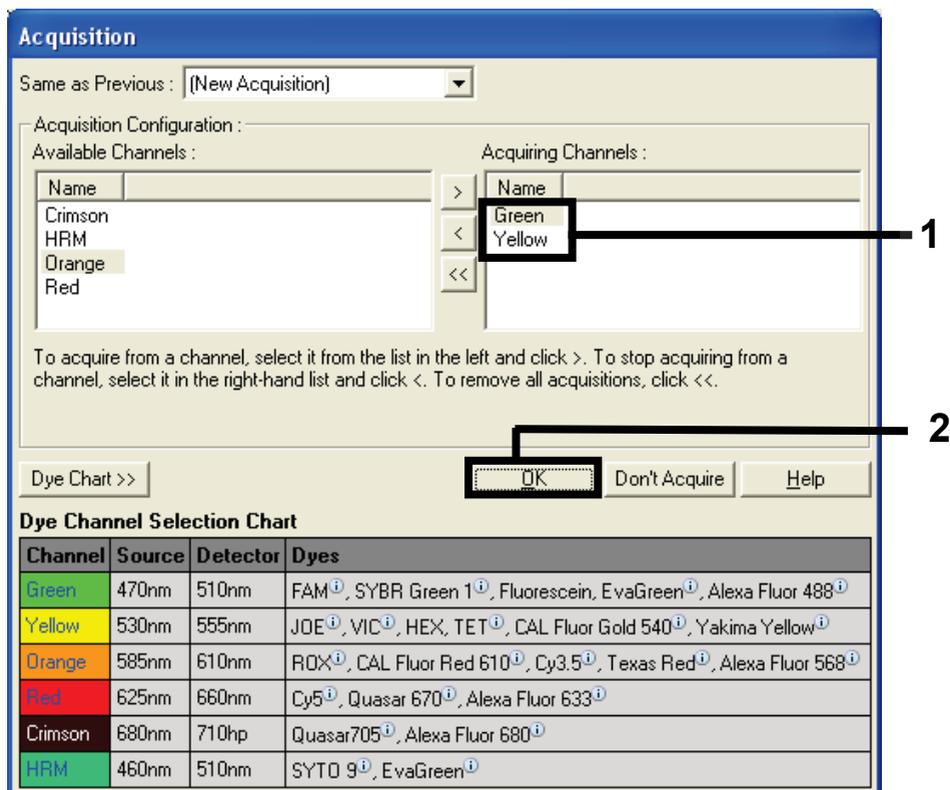
Figure 33. Acquiring at cycling step of 60°C.

11. Highlight the third step and click **–** to delete. Click **OK** (Figure 36).
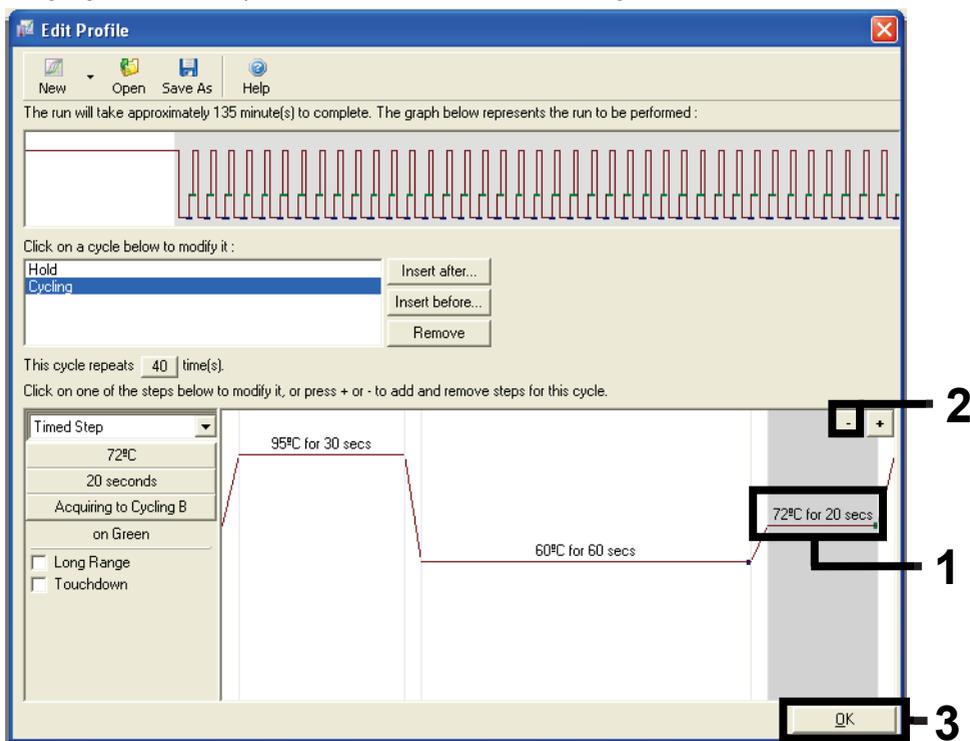
Figure 34. Removal of extension step.

12. In the next window, click **Gain Optimization** (Figure 37).
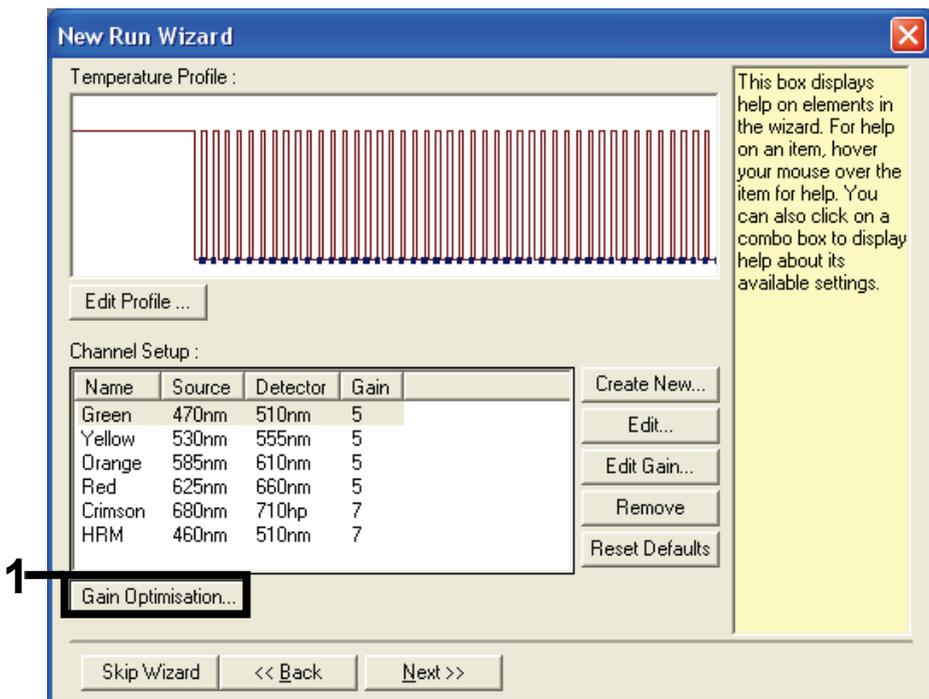
Figure 35. Gain Optimization.

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13. Click **Optimize Acquiring**. Channel settings are displayed for each channel. Click **OK** to accept these default values. (Figure 38).
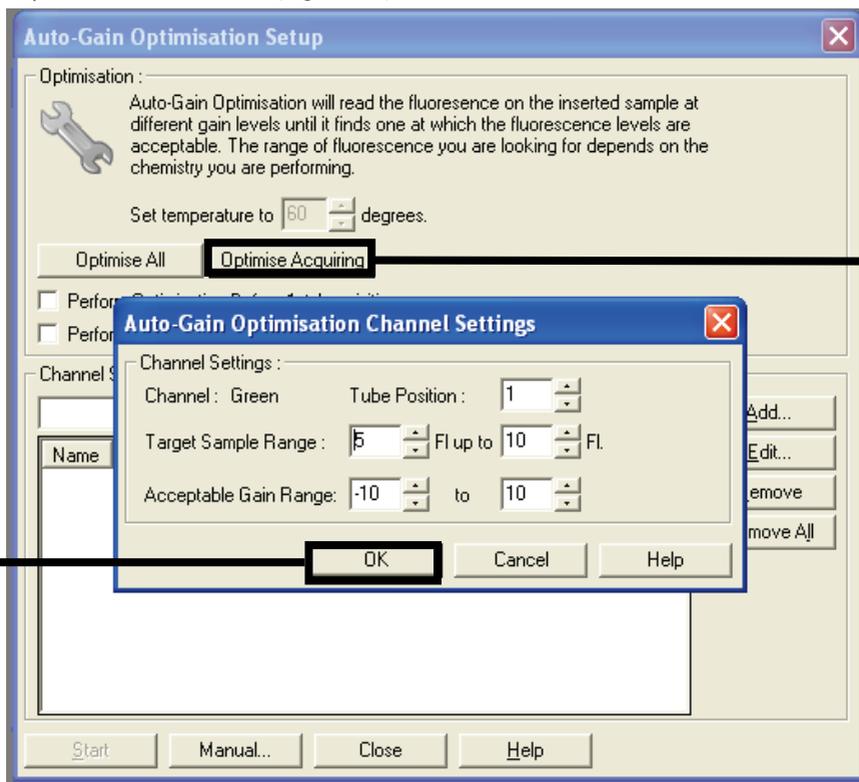
Figure 36. Auto-gain Optimization for the green channel.

14. Check the **Perform Optimization before 1st Acquisition** box then, click **Close** to return to the wizard (Figure 39).
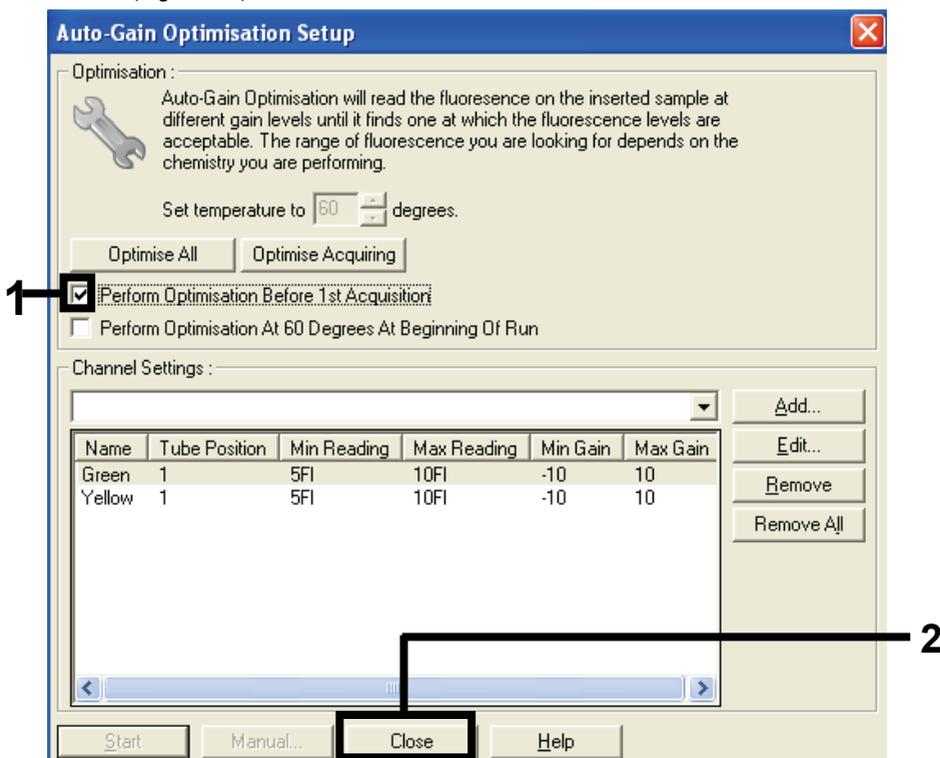
Figure 37. Selection of green and yellow channels.

15. Click **Next**. Then, click **Save** to save the template in an appropriate location.

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 5plex HRM5plex HRM 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: DNA sample assessment.
- Set up the PCR run on the Rotor-Gene Q MDx 5plex HRM5plex HRM 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: *therascreen* KRAS PCR RGQ setup

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

2. Create the temperature profile according to Protocol: Creating a temperature profile.

   Ensure that the correct rotor is selected, and check the **Locking Ring Attached** box. Click **Next** (Figure 40).
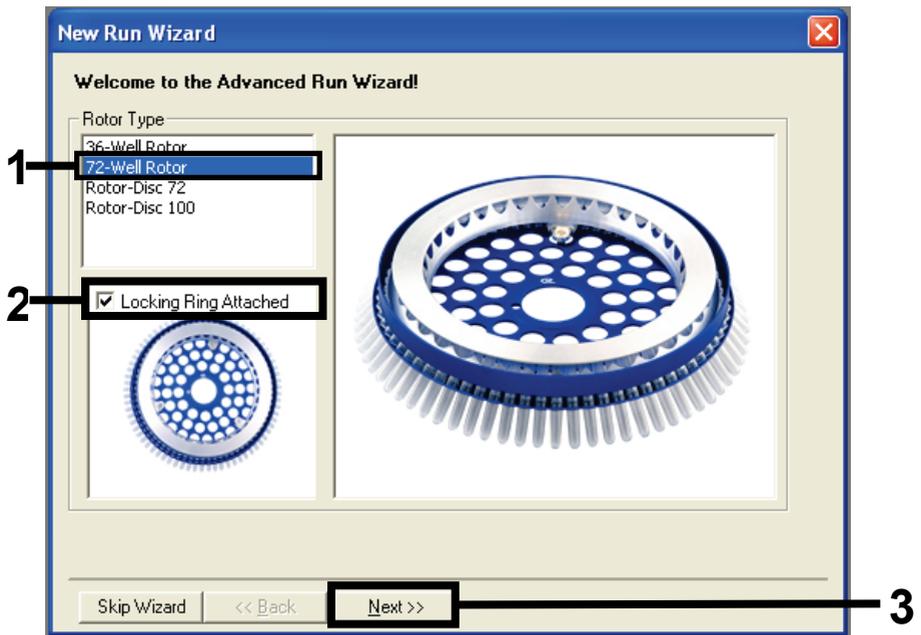
Figure 38. The New Run Wizard dialog box and welcome screen. 1 = Rotor type, 2 = Locking Ring Attached box, 3 = Next.

3. Enter the name of the operator. Add any notes and check that the **Reaction Volume** field is set to **25** and the **Sample Layout** field box contains the value **1, 2, 3…**. Click **Next** (Figure 41).

Figure 39. The New Run Wizard dialog box. 1 = Operator and Notes fields, 2 = Reaction Volume and Sample Layout fields, 3 = Next.

4. Leave all values as it is in the next window. No editing is required as the temperature profile has been created according to the instructions in Protocol: Creating a temperature profile. Click **Next** (Figure 42).
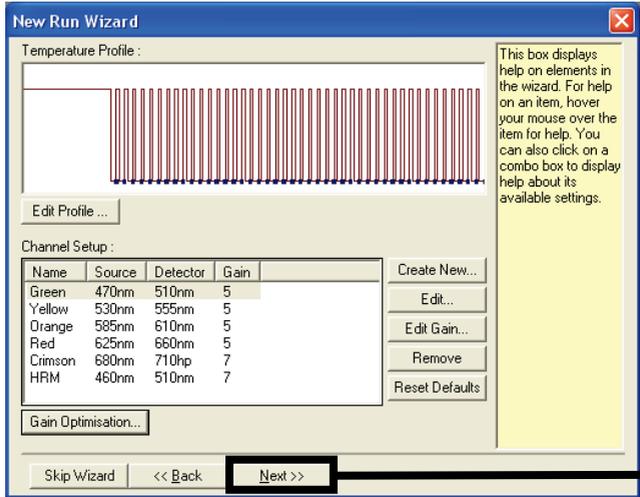
Figure 40. The New Run Wizard dialog box and temperature editing screen. 1 = Next.

5. Review the summary and click **Start Run** to save the run file and start the run (Figure 43).
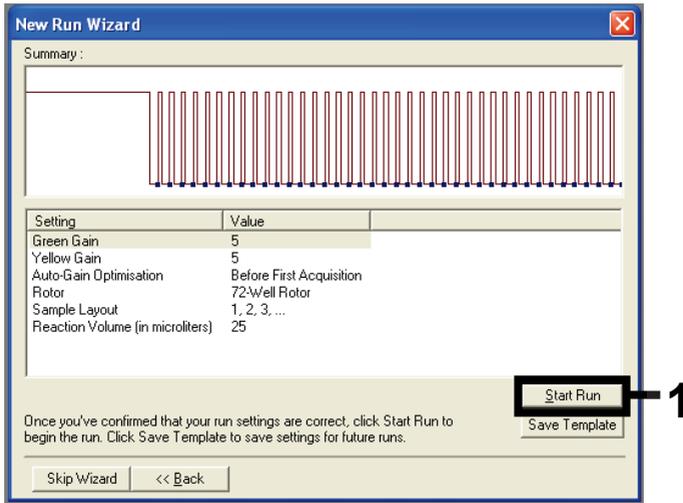
Figure 41. The New Run Wizard dialog box. 1 = Start Run.

Note: 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.

If you click **Finish and Lock Samples,** you can no longer edit the sample names. You 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.

6. After the run is complete, analyze data according to sections Sample assessment data analysis or KRAS mutation detection analysis, as appropriate.

7. If quantification reports are required, click the **Reports** icon of the toolbar in the Rotor-Gene Q run file.

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 5plex HRM with a 72-well rotor. The *therascreen* KRAS Assay Package is available separately on CD (cat. no. 9023675).

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 3.0.3 (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. Double-click the file and extract the file inside the archive.
3. Double-click **therascreen_KRAS_Assay_Package_3.0.3.exe** to start the installation.

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 3.0.3. Cat. no. 9023675.

2. Insert the CD into the CD drive of the laptop connected to the Rotor Gene Q MDx 5plex HRM instrument.

3. Double-click **therascreen_KRAS_Assay_Package_3.0.3.exe** or **therascreen_KRAS_Assay_Package_1.0.12.exe** to start the installation

   The setup wizard will appear.

4. Click **Next** to continue (Figure 46).
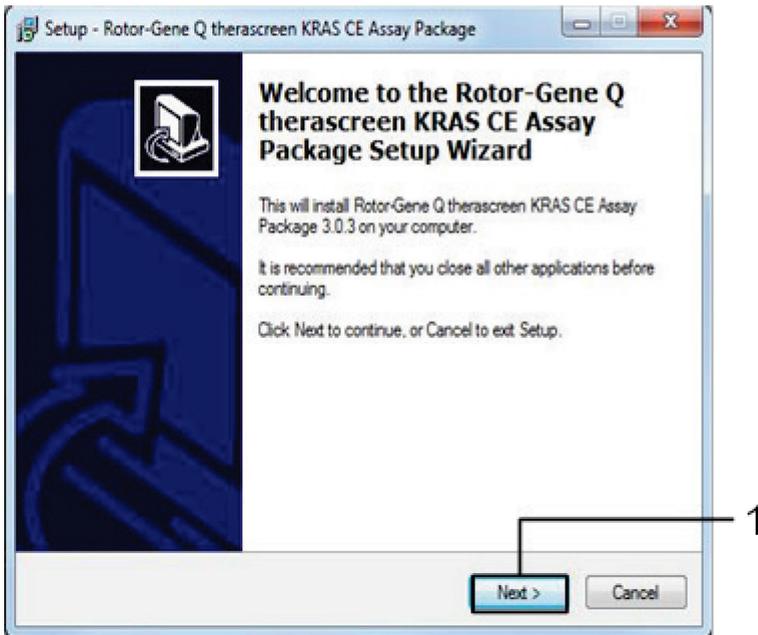
Figure 42. The Setup dialog box. 1 = Next.

5. Read the License Agreement in the License Agreement dialog box and check the **I accept the agreement box**. Click **Next** to continue (Figure 47).
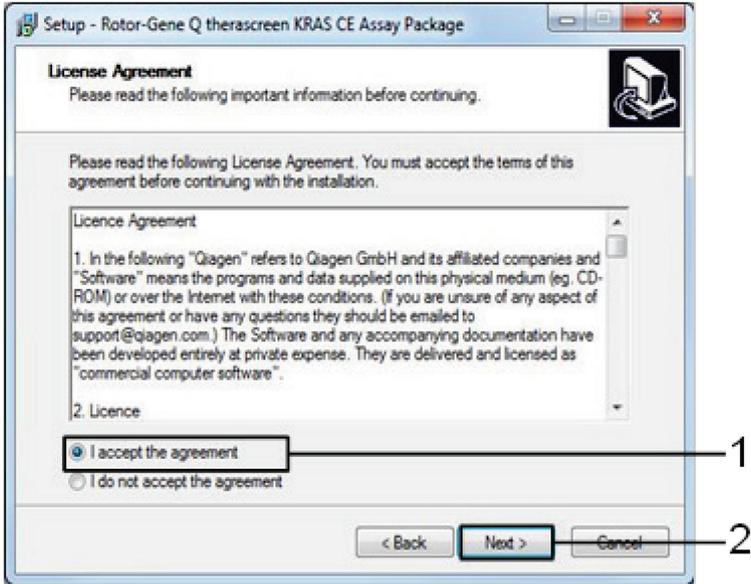
Figure 43. The License Agreement dialog box. 1 = I accept the agreement statement, 2 = Next.

The template setup will start automatically.

6. In the final Setup window, click **Finish** to exit the setup wizard. (Figure 48).
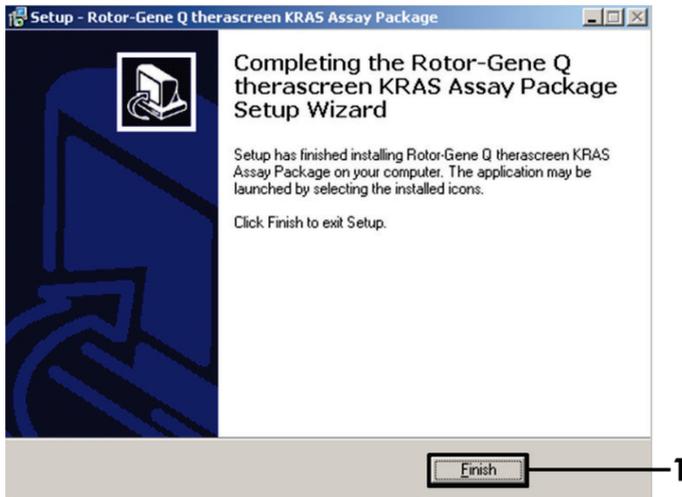
Figure 44. Completing the wizard.

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

Ordering Information

Product	Contents	Cat. no.
therascreen KRAS RGQ PCR Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, Water, *Taq* DNA Polymerase	874011
therascreen KRAS Assay Package CD (version 3.0.3)	Software protocol package for use with the *therascreen* KRAS RGQ PCR Kit and the QIAGEN Rotor-Gene Q MDx 5plex HRM instrument with a 72-well rotor	9023675
Rotor-Gene Q and accessories		
Rotor-Gene Q MDx 5plex HRM	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.

Document Revision History

Date	Changes
R4, January 2019	Addition of Authorized Representative (front cover)
	Updated Symbols section
	Updated template
R5, November 2019	Change in legal manufacturer (cover page)
	Removed EC + REP symbol from cover page and Symbols section
	Adaption of instrument name from Rotor-Gene Q MDx to Rotor-Gene Q MDx 5plex HRM to align with name on the instrument label
	Updated the Detection of KRAS mutations protocol to include additional step in preparing master mixes
	Corrected values in Frequency and 95% Confidence Interval columns in Table 9.
	Updated CRC overall agreement percentage from 96.4% to 96.82%
	Corrected values in LOD C_{95} column in Table 14
R6, November 2020	Corrected the therascreen KRAS Assay Package version to 3.0.3
	Updated this document all through-out regarding the availability for download of the assay package at **www.qiagen.com**
	Added a note throughout the document regarding actions related to flags for NSCLC indication updated to ensure correct functioning of the kit
	Added a note throughout the procedures in this document about correct reagent mixing
	Updated General precautions section
	Added a note for correct placement of Rotor-Gene Q MDx 5plex HRM instrument
	Added a note regarding the use of dry scalpels
	Updated Protocol: DNA sample assessment to add additional important points before starting
	Updated Protocol: Detection of KRAS mutations section to add additional important points before starting and a note for NSCLC samples to interpret several flags as Invalid
	Updated Rotor-Gene Q therascreen KRAS Assay Package flags section to add tables to illustrate RGQ flags, their meanings, and recommended actions
	Updated Interpretation of Results regarding actions related to flags.
	Updated Troubleshooting Guide section to add tables to illustrate RGQ flags, their meanings, and recommended actions
	Updated Limitations section to add information about NSCLC samples

Date	Changes
	Revised Performance Characteristics section to update data in tables
	Updated DNA input and linearity section
	Updated Repeatability and reproducibility section
	Updated Sample handling variability section
	Clinical Performance Section added
	Updated Sample assessment data analysis section to revise recommended solution for sample control assay C_T of >32
	Marking for European conformity added to Symbols section
	Updated Interpretation of Results (Manual) regarding actions related to flags.
	Updated the Protocol: Detection of KRAS mutations section to include steps on how to prepare the master mixes

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