May 2021

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

For in vitro diagnostic use

For prescription use only

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

For use with the QIAamp® DSP DNA FFPE Tissue Kit

Version 1

IVD

REF   870021

QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

R10 **MAT**   1123716EN

Sample to Insight

QIAGEN

Contents

therascreen KRAS RGQ PCR Kit Instructions for Use (Handbook) 05/2021

Intended Use

The *therascreen*® KRAS RGQ PCR Kit is a real-time qualitative PCR assay used on the Rotor-Gene Q MDx instrument for the detection of seven somatic mutations in the human KRAS oncogene, using DNA extracted from formalin-fixed, paraffin-embedded (FFPE), colorectal cancer (CRC) tissue and non-small cell lung cancer (NSCLC) tissue. The test is intended as a companion diagnostic to identify patients who may benefit from treatment with the targeted therapies listed in Table 1 in accordance with the approved therapeutic product labeling.

Table 1. Companion diagnostic indications

Indication	Biomarker	Therapy
Colorectal cancer	*KRAS* wild-type (absence of mutations in codons 12 and 13)	Erbitux® (cetuximab) Vectibix® (panitumumab)
Non-small cell lung cancer (NSCLC)	*KRAS* G12C	LUMAKRAS™ (sotorasib)

Summary and Explanation

Mutations in the *KRAS* oncogene are frequently found in human cancers (1–3). The presence of these mutations correlates with a lack of response to certain EGFR inhibitor cancer therapies in patients with metastatic colorectal cancer (mCRC) (4, 5–13). Such mutations in the *KRAS* oncogene are present in around 40% of mCRC cases (14).

The *KRAS* G12C mutation specifically results in the expression of a form of the KRas protein in which the Glycine residue at amino acid position 12 is replaced by a Cysteine residue. In NSCLC patients, this indicates potential eligibility for treatment with the targeted KRas inhibitor LUMAKRAS™ (sotorasib) (Amgen, data on file). This mutation is estimated to be present in around 13% of NSCLC cases (15).

Using Scorpions® (16) and ARMS (Allele Refractory Mutation System) technologies (17, 18), the *therascreen* KRAS RGQ PCR Kit enables the detection of seven mutations in codons 12 and 13 of the *KRAS* oncogene against a background of wild-type genomic DNA (Table 2). Based on data in the COSMIC database (2012 v59), the seven mutations detected by the *therascreen* KRAS RGQ PCR Kit account for >97% of all reported *KRAS* mutations in CRC patients (19).

Table 2. 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:
(**www.sanger.ac.uk/genetics/CGP/cosmic**).

Principle of the Procedure

The *therascreen* KRAS RGQ PCR Kit provides 8 separate PCR amplification reactions: 7 mutation-specific reactions in codons 12 and 13 of exon 2 of the *KRAS* oncogene and a wild-type control in exon 4. The principal components of the kit are explained below.

Mutation reaction mixes

Each mutation-specific reaction mix uses a mutation-specific Amplification Refractory Mutation System (ARMS) primer to selectively amplify 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 bifunctional 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.

Control reaction

The Control Reaction Mix (tube 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.

Internal control

Each of the 8 reaction mixes contains an internal control designed to detect failure of the reaction (e.g., due to the presence of inhibitors). The internal control employs a non-KRAS related oligonucleotide target sequence, an unlabeled primer and a Scorpions primer labeled with hexachlorofluorescein (HEX™) in order to distinguish it from the FAM labeled Scorpions in the control and mutation reactions.

Positive control

therascreen KRAS RGQ PCR Kit Instructions for Use (Handbook) 05/2021

The *therascreen* KRAS RGQ PCR Kit also contains the KRAS Positive Control (tube PC). The positive control is a mixture of synthetic oligonucleotides representing each of the mutations detected by the kit. Detection of the positive control confirms the proper functioning of each of the reaction mixes in the kit.

Negative control

The *therascreen* KRAS RGQ PCR Kit contains nuclease-free water for no template control (tube NTC) to be used for the "no template control" reaction. The no template control serves as a negative control and assesses potential contamination during assay setup.

Taq DNA polymerase

Taq DNA polymerase is the enzyme for the polymerase chain reaction used by the *therascreen* KRAS RGQ PCR Kit.

Platform and software

The *therascreen* KRAS RGQ PCR Kit is specifically designed for use with the Rotor-Gene Q MDx instrument installed with the *therascreen* KRAS Assay Package, available separately on CD.

- Refer to "Materials Required but Not Provided," page 17, for compatible RGQ Software and *therascreen* KRAS Assay Package versions.
- Refer to the instrument user manual for information concerning the instrument.
- Refer to "Appendix: Installation of the Rotor-Gene Q *therascreen* KRAS Assay Package," page 139, for instructions on installing the Rotor-Gene Q *therascreen* KRAS Assay Package.

The Rotor-Gene Q MDx (US) instrument must be maintained according to the requirements in the instrument user manual.

Run parameters

The Rotor-Gene Q MDx (US) instrument is programmed for different cycle parameters, or "runs," by the *therascreen* KRAS Assay Package. The *therascreen* KRAS Assay Package consists of two templates:

● The "*therascreen* KRAS QC Locked Template" (for DNA sample assessment)

● The "*therascreen* KRAS Locked Template" (for detection of KRAS mutations)

These assay templates contain the PCR run parameters and calculate the results. The same run parameters are used for both the DNA sample assessment with the Control Reaction Mix and for detection of KRAS mutations using the mutation reaction mixes.

1. Hold at 95°C for 15 minutes to activate the *Taq* DNA polymerase.
2. PCR for 40 cycles of 95°C for 30 seconds to denature, and 60°C for 1 minute to anneal and extend.

The PCR cycle at which the fluorescence from a particular reaction crosses the predefined threshold value given by *therascreen* KRAS Assay Package is defined as the C_T value.

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

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

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

Based on predetermined analytical C_T and ΔC_T values, the Rotor-Gene Q software qualitatively determines the mutation status of the DNA samples and reports which samples contain which mutation.*

Reagents

Reaction mixes are duplex, containing FAM-labeled reagents to detect targets and HEX-labeled internal control. The reaction mixes and positive control reagents contain Tris EDTA (ethylenediaminetetraacetic acid) buffer, and the positive control contains the carrier nucleic acid Poly A RNA.

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

Table 3. Reagents supplied in the *therascreen* KRAS RGQ PCR Kit

Reagent	Ingredients	Quantity
Control Reaction Mix	Control Unlabeled Primer, Control Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	2 tubes x 600 µl
12ALA Reaction Mix	12ALA ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl
12ASP Reaction Mix	12ASP ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl
12ARG Reaction Mix	12ARG ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl
12CYS Reaction Mix	12CYS ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl

Table continued on next page

Table continued from previous page
Table 3. Reagents supplied in the *therascreen* KRAS RGQ PCR Kit

Reagent	Ingredients	Quantity
12SER Reaction Mix	12SER ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl
12VAL Reaction Mix	12VAL ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl
13ASP Reaction Mix	13ASP ARMS Primer, Scorpions Primer, Internal Control Unlabeled Primer, Internal Control Scorpion Primer, Internal Control Template, Deoxynucleotide Triphosphates, Magnesium Chloride, Tris EDTA Buffer, PCR Buffer	1 tube x 600 µl
KRAS Positive Control	Control Template, 12ALA Template, 12ASP Template, 12ARG Template, 12CYS Template, 12SER Template, 12VAL Template, 13ASP Template, Poly A RNA, Tris EDTA Buffer	1 tube x 250 µl
Taq DNA Polymerase	*Taq* DNA Polymerase: 50% glycerol/water	1 tube x 80 µl
No template control	Nuclease-free water	1 tube x 1.9 ml
For template dilution	Nuclease-free water	1 tube x 1.9 ml

Materials Provided

Kit contents

therascreen KRAS RGQ PCR Kit			**(24)**
Catalog number			**870021**
Number of preps			**24**
Color	**Identity**	**Tube ID**	**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
Gray	12VAL Reaction Mix	7 12VAL	600 µl
Blue	13ASP Reaction MixP	8 13ASP	600 µl
Beige	KRAS Positive Control	9 PC	250 µl
Mint	*Taq* DNA Polymerase	Taq	80 µl
White	Nuclease-free water for no template control	NTC	1.9 ml
White	Nuclease-free water for dilution	Dil.	1.9 ml
therascreen *KRAS RGQ PCR Kit Instructions for use (Handbook)*			1

Materials Required but Not Provided

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

Make sure that instruments have been checked and calibrated according to the manufacturer's recommendations.

- 0.1 ml Strip Tubes and Caps, for use with 72-well rotor (QIAGEN, cat. no. 981103 or 981106)
- Sterile microcentrifuge tubes for preparing master mixes
- Sterile pipet tips with aerosol barriers
- Permanent marker
- Rotor-Gene Q MDx (US) instrument with 72-well rotor (QIAGEN, cat. no. 9002035 and 9001580)

  Rotor-Gene Q software version 2.3 with *therascreen* KRAS Assay Package CD version 3.1.1 (QIAGEN, cat. no. 9023818)

  Note: The Rotor-Gene Q *therascreen* KRAS Assay Package software will only work with software version 2.3.

  Note: Previous versions of the assay package are not compatible with the new software version 2.3.
- Loading Block 72 x 0.1 ml Tubes, aluminum block for manual reaction setup (QIAGEN, cat. no. 9018901)
- Dedicated pipets (adjustable) for sample preparation
- Dedicated pipets (adjustable) for PCR master mix preparation
- Dedicated pipets (adjustable) for dispensing of template DNA

- Thermomixer, heated orbital incubator, heating block, or water bath capable of incubation at 56°C, 70°C and 90°C

- Benchtop centrifuge with rotor for 1.5 ml tubes

- Benchtop vortexer

- QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404; see "DNA extraction," page 25)

Warnings and Precautions

Warnings

For in vitro diagnostic use

For prescription use only

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

For use with QIAamp DSP DNA FFPE Tissue Kit

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

Precautions

The user must always adhere to the following precautions 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 *therascreen* KRAS RGQ PCR Kit.

- All reagents supplied in the *therascreen* KRAS RGQ PCR Kit are intended to be used solely with the other reagents supplied in the same *therascreen* KRAS RGQ PCR Kit. Do not substitute the reagents in the *therascreen* KRAS RGQ PCR Kit or between *therascreen* KRAS RGQ PCR Kits, as this may affect performance.
- Only use the *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.
- Refer to the *Rotor-Gene Q MDx (US) User Manual* for additional warnings, precautions, and procedures.
- Do not use expired or incorrectly stored components.

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 (US) instrument until the run has finished.

Note: Do not open 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, if the outer packaging has been opened during transit or the shipment does not contain a packing note, Instructions for Use or the reagents, please contact QIAGEN Technical Services or local distributors (visit www.qiagen.com).

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

Once opened, reagents can be stored in their original packaging at –30 to –15°C for 12 months or until the stated expiration date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of 6 freeze–thaw cycles.

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

- 7 hours if stored at room temperature

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

- 18 hours if stored in the refrigerator (2–8°C)

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

Note: Scorpions (as with all fluorescently labeled molecules) in the reaction mix reagents are light sensitive. Protect control and reaction mix reagents from light to avoid photo bleaching.

Reagents in the *therascreen* KRAS RGQ PCR Kit are diluted optimally and no further purification or treatment is required prior to their use in analysis as directed by the therascreen *KRAS RGQ PCR Kit Instructions for Use (Handbook)* (this document).

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

Specimen Handling and Storage

Specimen handling

The *therascreen* KRAS RGQ PCR Kit is for use on DNA samples extracted from FFPE tumor tissue collected from CRC or NSCLC patients. Tumors are heterogeneous in terms of both genotype and phenotype. Mutation-positive tumors can contain wild-type DNA and similarly histology can show regions of non-tumor tissue. All tissue samples should be treated as potentially hazardous.

Preparing tissue specimens 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.
- Use a trained individual (e.g., a pathologist) to 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 two 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.

Preparing tissue specimens 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 an 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 for up to 8 weeks before use.

Protocol: DNA extraction

DNA extraction for CRC samples

Use the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) 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 DSP DNA FFPE Tissue Kit. Do not use any other DNA extraction product.

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

- See the *QIAamp DSP DNA FFPE Tissue Kit Handbook* for preparation of specimens prior to DNA extraction.
- The QIAamp DSP DNA FFPE Tissue Kit must be used manually only. Do not use the automated protocol for the QIAcube® described in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.
- Do not use the RNase step described in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.
- Do not use the QIAGEN Deparaffinization Solution provided in the QIAamp DSP DNA FFPE Tissue Kit. Use only the xylene/ethanol method for deparaffinization described in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*.
- Use molecular biology grade ethanol for all required steps.*
- Use 1 slide per extraction.

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

- Proteinase K digestion (step 11 in the *QIAamp DSP DNA FFPE Tissue Kit Handbook*) must be performed for 1 hour.

- The samples must be eluted using 200 µl of elution buffer (Buffer ATE) from the QIAamp DSP DNA FFPE Tissue Kit.

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 DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) 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 DSP DNA FFPE Tissue Kit. Do not use any other DNA extraction product.

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

- Use 2 x 5 µm sections per extraction.

- The QIAamp DNA FFPE Tissue Kit must be used manually only.

- Do not use the RNase step in the *QIAamp DNA FFPE Tissue Kit Handbook*.

- Do not use the QIAGEN deparaffinization solution provided in the QIAamp DNA FFPE Tissue Kit. Use only the xylene/ethanol method for deparaffinization described in the *QIAamp DNA FFPE Tissue Kit Handbook.*

- Proteinase K digestion (step 11 in the *QIAamp DNA FFPE Tissue Kit Handbook*) must be performed for 1 hour.

- Add 60 µl elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit and incubate for 2.5 minutes at room temperature.

- Centrifuge at full speed for 1 minute.

- Add a further 60 µl elution buffer (ATE) from the QIAamp DNA FFPE Tissue Kit and incubate for 2.5 minutes at room temperature.

- Centrifuge at full speed for 1 minute.

- 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.

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 available.

● Use the Control Reaction Mix (tube CTRL) to assess the DNA prior to testing.

● 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.

Things to do before starting

● Make sure that the Rotor-Gene Q *therascreen* KRAS Assay Package is installed before first use of the Rotor-Gene Q MDx (US) instrument (see "Appendix: Installation of the Rotor-Gene Q *therascreen* KRAS Assay Package," page 139).

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

1. Completely thaw the Control Reaction Mix (tube CTRL), 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.

Thaw time		Storage temperature after PCR setup	Maximum PCR setup and storage time
Minimum	Maximum		
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 (US) 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," page 21). 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 briefly to collect the contents at the bottom of the tube.

3. Prepare sufficient control assay master mixes (Control Reaction Mix [tube CTRL] plus *Taq* DNA polymerase [tube 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 below. Include reagents for 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 control or sample.

Component of control assay master mix	Volume required for master mix
Control Reaction Mix (tube CTRL)	19.76 µl x (n + 1)*
Taq DNA Polymerase (tube Taq)	0.24 µl x (n + 1)
Total volume	**20 µl per reaction**

* 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 24 (plus controls) as 24 is the maximum number of samples which can fit on a run.

Note: When preparing the control assay master mix, the required volume of the Control 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 below. 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.

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	–	–	–	–	–	–

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

6. Immediately add 20 μl control assay master mix to each PCR strip tube. Refer to the table 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 (tube 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 (tube PC) to the PC tube (tube position 1) and cap the tube.

   Each tube should contain a total reaction volume of 25 μl (20 μl of control assay master mix 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 (US) instrument.

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

12. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout using the 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 (US) instrument is maintained.

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

14. Start the Rotor-Gene Q software and open the template at the same time by double-clicking on the **therascreen KRAS QC Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q MDx (US instrument.

15. The **Setup** tab appears (1) as the default. Ensure that the locking ring is properly attached and check the **Locking Ring Attached** box (2). Close the lid of the Rotor-Gene Q MDx (US) instrument.
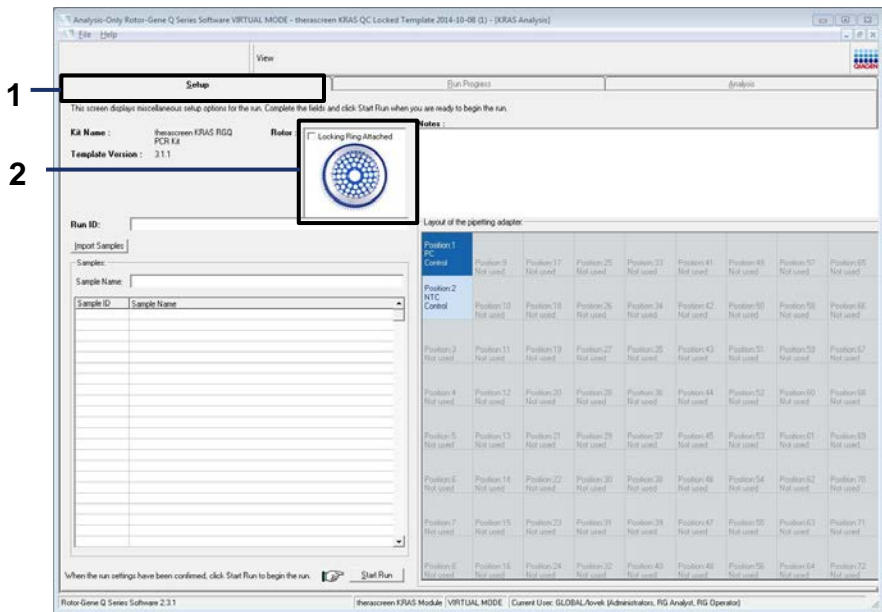
16. Enter the run ID in the **Run ID** dialog field (1 in example below) according to your local naming convention.
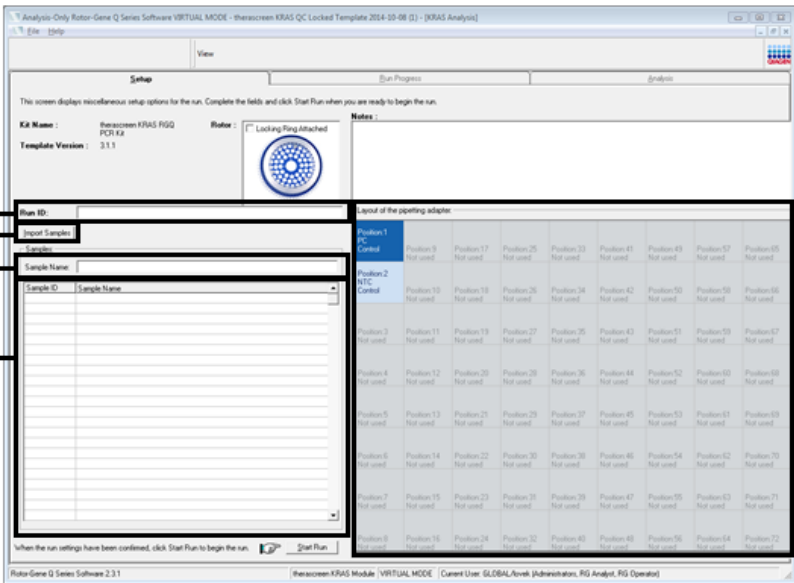
17. Enter the sample name in the **Sample Name** dialog field (3) according to your local naming convention and press the return key. This will add the sample name to the sample list below (4) and assign the sample a **Sample ID** (1, 2, 3, etc.). In addition, the **Layout of the pipetting adapter** (5) panel on the right side will update to include the sample name.

Important: Use caution when entering sample names to avoid mixing up the sample IDs. 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 (2). 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 the sample name is in the sample position.

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

18. Repeat step 17 to enter the names of all additional samples.

    Note: To edit a sample name, click on the sample name in the sample list (4) and the selected sample will appear in the **Sample Name** dialog field (3) above. Edit the sample name according to your local naming convention and press the return key to update the name. The additional sample names appear in the **Layout of the pipetting adapter** panel (5).

19. When all sample names have been entered, verify they are correct. Add any additional information in the **Notes** dialog field (1) if necessary and then click the **Start Run** button (2).
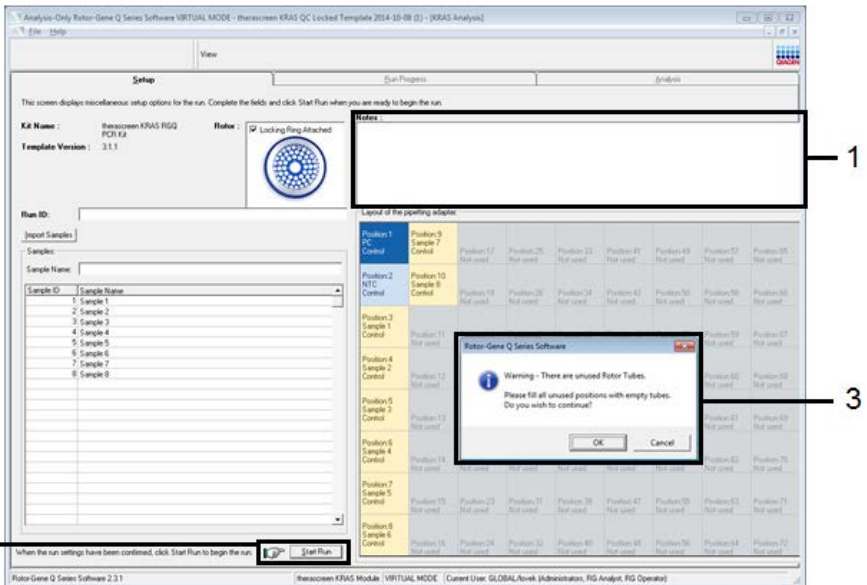
Note: If any rotor position is unused, a warning will appear (3 and example below) to remind the user that all unused positions on the rotor must be filled

with a capped, empty tube. Check that all unused rotor positions are filled with
a capped, empty tube and click **OK** (1 in the example below) to proceed.
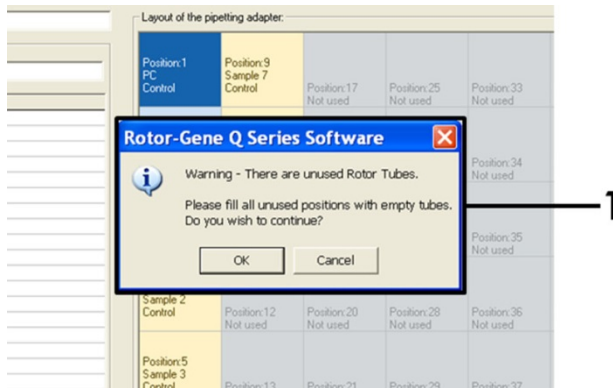
20. A **Save As** window appears. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location and click the **Save** button.
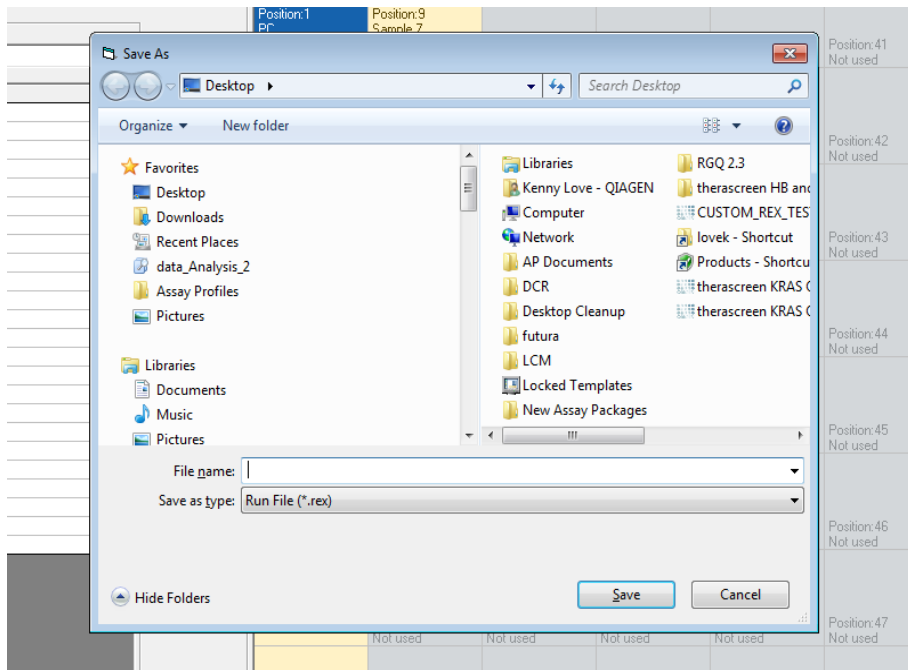
21. The PCR run starts.

Note: When the run starts, the **Run Progress** tab will open automatically to show the temperature trace and remaining run time.
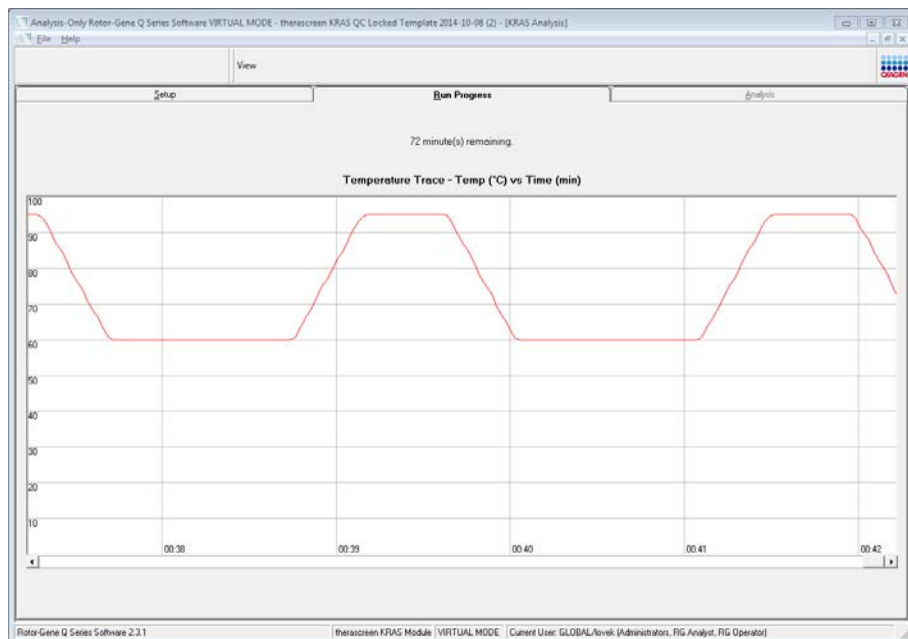
After the run is finished, the **Analysis** tab will open automatically. If the **Analysis** tab fails to open, click the **Analysis** tab.
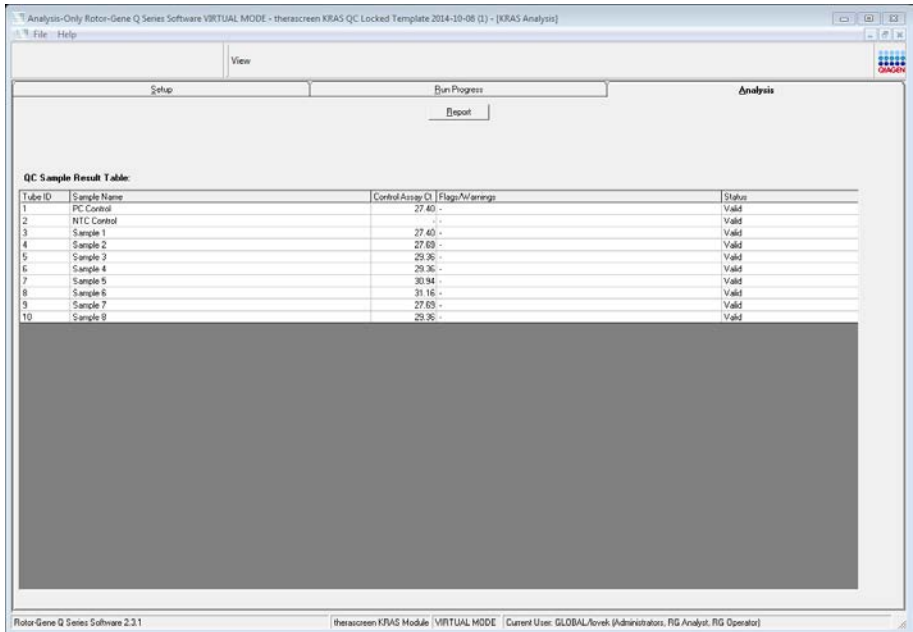
22. Control results are reported in the **QC Sample Result Table**.

- Run controls (PC Control and NTC Control, tube positions 1 and 2 respectively):

    If the results are within acceptable ranges, each will display a status of "Valid."

    If the results are not within acceptable ranges, an "Invalid" result will appear.

- Sample control reaction C_T is >32.00: an "Invalid" result will display.

    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 "Rotor-Gene Q *therascreen* KRAS Assay Package flags," page 56, and "Troubleshooting Guide," page 63).

- Sample control reaction C_T is <21.92: an "Invalid" result will display.

The DNA concentration is too high for mutation analysis.

Dilute with nuclease-free water for dilution (tube 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 is 21.92–32.00, (21.92 ≤Control C_T ≥ 32.00): a "Valid" result will display.

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.

23. Produce Report files by clicking the **Report** button. The **Report Browser** window will appear. Select **KRAS Analysis Report** under **Templates** and then click the **Show** button.
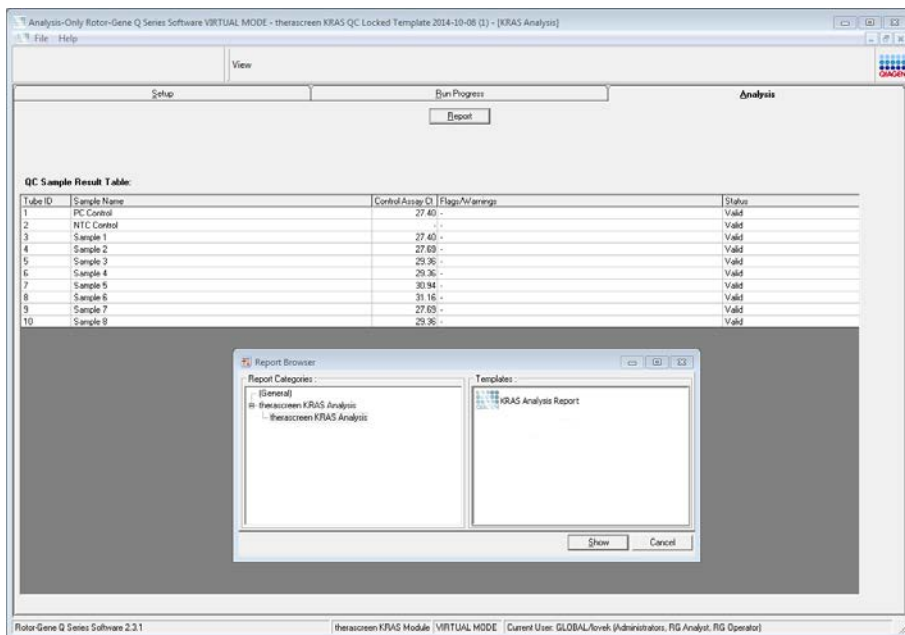
Note: Reports can be saved to an alternative location in Web Archives format by clicking the **Save As** button on the top left corner of each report.

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.

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.

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.

Thaw time		Storage temperature after PCR setup	Maximum PCR setup and storage time
Minimum	Maximum		
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," page 21). 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 briefly to collect the contents at the bottom of the tube.

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

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 below. 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.

| | Controls | | Sample number | | | | | | |
Assay	PC	NTC	1	2	3	4	5	6	7
Control	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
12ASP	8	16	24	32	40	48	56	64	72

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

    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 5 µl of nuclease-free water for no template control (tube NTC) to the NTC tubes (tube positions 9–16) and cap the tubes.

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

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

9. Using a permanent marker, mark the lids of the first tubes in the lowest numerical position in each PCR 4-strip tube (e.g., positions 1, 5, and 9, etc.) to show the orientation to load the tubes into the 72-well rotor of the Rotor-Gene Q MDx instrument.

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

11. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout using the marks for orientation.

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

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

13. Start the Rotor-Gene Q software and open the template at the same time by double-clicking on the **therascreen KRAS Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q MDx (US) instrument.
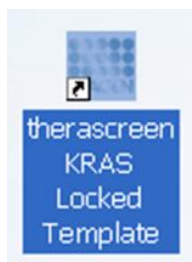
14. The **Setup** tab appears (1) as the default. Ensure that the locking ring is properly attached and check the **Locking Ring Attached** box (2). Close the lid of the Rotor-Gene Q MDx (US) instrument.
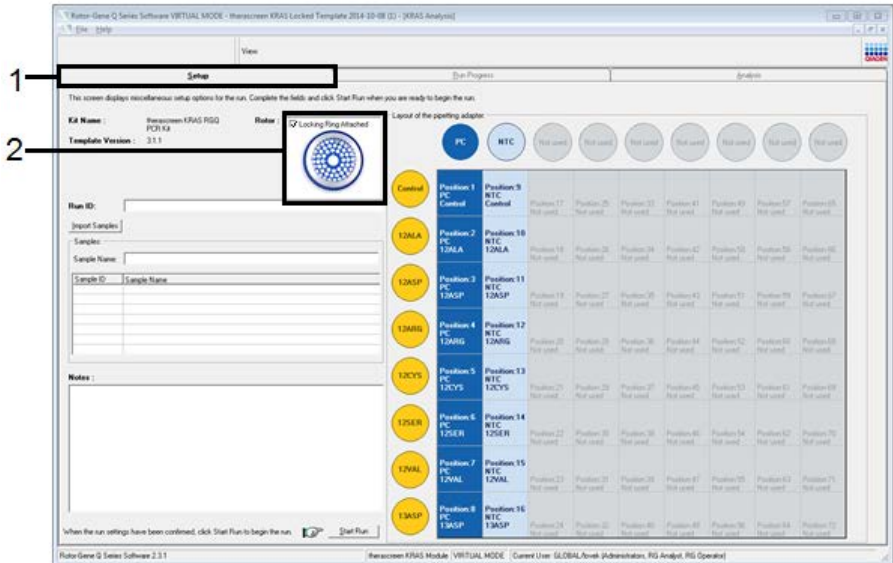
15. Enter the run ID in the **Run ID** dialog field (1 in the example below) according to your local naming convention.

16. Enter the sample name in the Sample Name dialog field (3) according to your local naming convention and press the return key.

Important: Use caution when entering sample names to avoid mixing up the sample IDs.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 (5) will update to include the sample name.

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 (2). 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 all eight assays in the column under the sample circle are highlighted (6).

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.

17. Repeat step 16 to enter the names of all additional samples.

Note: To edit a sample name, click on the sample name in the sample list (4) and the selected sample will appear in the **Sample Name** dialog field (3) above. Edit the sample name according to your local naming convention and press the return key to update the name.

18. When all sample names have been entered, verify they are correct. Add any additional information in the **Notes** dialog field (1 in the example below) if necessary and then click the **Start Run** button (2).
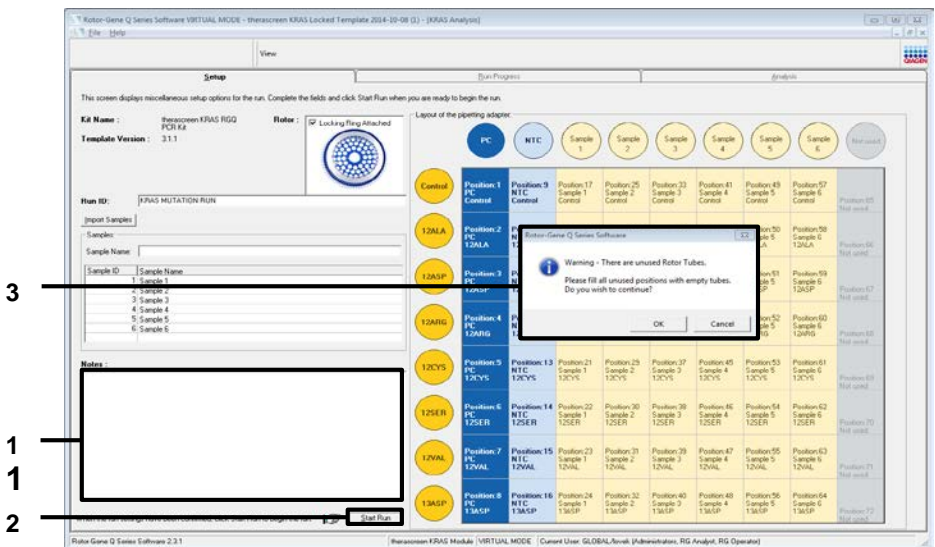
Note: If any rotor position is unused, a warning will appear (3 above and 1 in example below) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube.
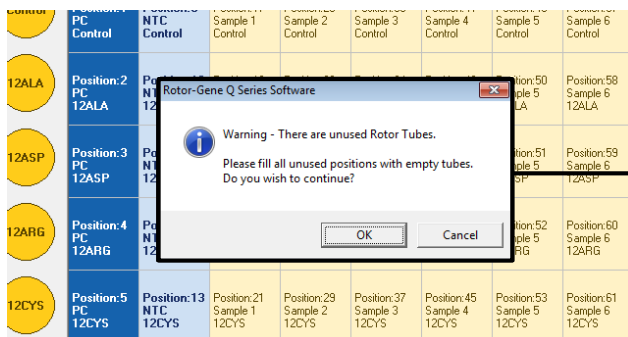
19. Check that all unused rotor positions are filled with a capped, empty tube and click **OK** to proceed.

20. A **Save As** window appears (1). Chose an appropriate file name (2) and click **Save** (3) to save the PCR run as a *.rex run file to the selected location.
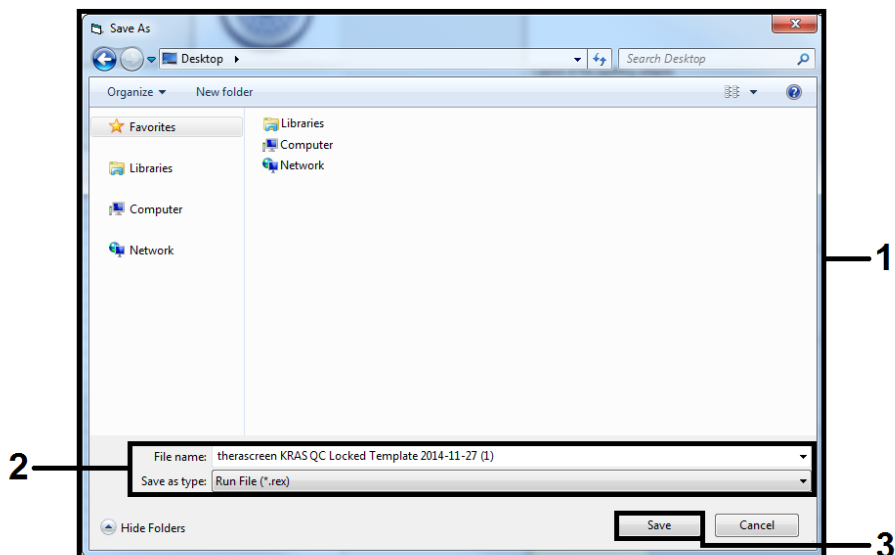
21. The PCR run starts.

Note: When the run starts, the **Run Progress** tab will open automatically to show the temperature trace and remaining run time.
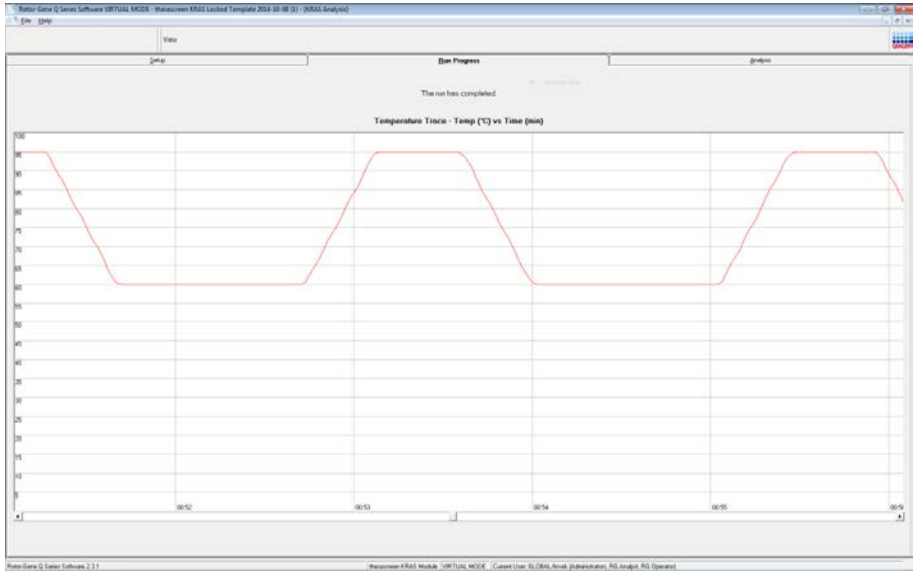
22. After the run is finished, the **Analysis** tab will open automatically.

    Note: If the **Analysis** tab fails to open, click the **Analysis** tab (1).

    Note: An explanation of the calculation method is presented in the "Analysis and mutation calls" section, page 54.
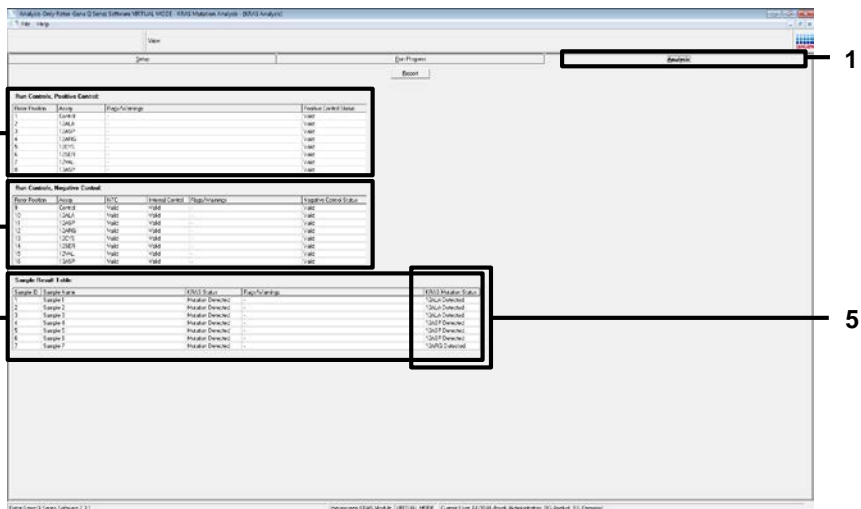
23. Assay results will be reported as follows:

- The **Run Controls, Positive Control** panel (2)

  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 (3)

  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 (4)

  Specific mutations will be reported for the mutation positive samples under the **KRAS Mutation Status** column (5).

24. Produce report files by clicking the **Report** button. The **Report Browser** window will appear. Select **KRAS Analysis Report** under **Templates**, and then click the **Show** button.
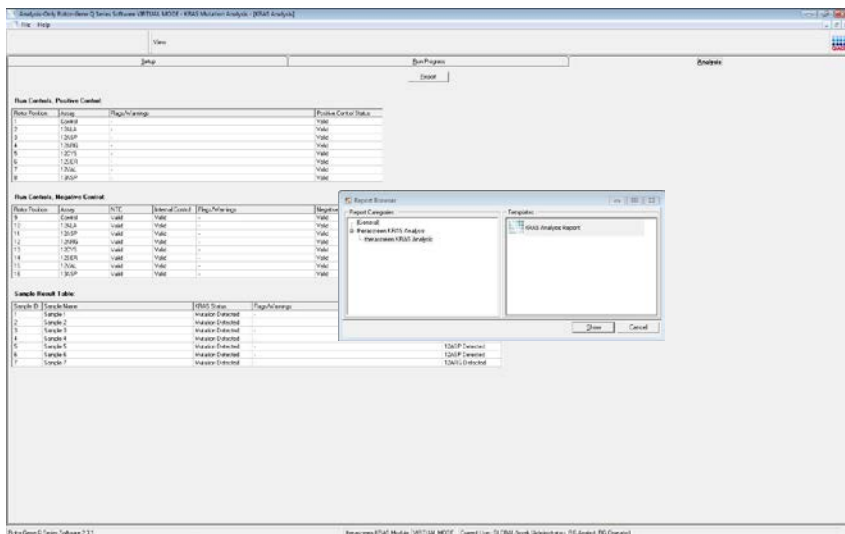
Note: Reports can be saved to an alternative location in Web Archives format by clicking the **Save As** button on the top left corner of each report.

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 two 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 Rotor-Gene Q *therascreen* KRAS Assay Package for both the green and yellow channels. 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. Other possible results that may be displayed are discussed in the "Protocol: DNA sample assessment" and "Troubleshooting Guide" sections of this Instructions for Use.

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

Rotor-Gene Q *therascreen* KRAS Assay Package flags

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.

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.

Flag	Meaning	Action
NTC_INVALID_CT	PCR run invalid — FAM invalid (smaller than limit) for negative control	Repeat the entire PCR run.
NTC_INVALID_DATA	PCR run invalid — Fluorescence data in negative control can't be interpreted	Repeat the entire PCR run.
SAMPLE_CTRL_INVALID_DATA	Sample invalid — Fluorescence data in sample control can't be interpreted	Setup new PCR run to repeat the relevant sample(s).
SAMPLE_CTRL_HIGH_CONC	Sample Invalid — FAM C_T too low in sample control	Dilute sample to increase control C_T value. This dilution should be calculated on the assumption that diluting 1:1 with the water supplied in the kit will increase the C_T by 1.0; once sample is diluted, setup new PCR run to repeat sample.

Flag	Meaning	Action
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.

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.

Flag	Meaning	Action
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.

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.

Flag	Meaning	Action
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.

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 kit components did not comply with the instructions given in "	Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit, if necessary.
b) Reagent Storage and Handling," page 21	
c) 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.

Quality Control

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

Limitations

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

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

The *therascreen* KRAS RGQ PCR Kit is used in a polymerase chain reaction (PCR) procedure. As with all PCR procedures, samples may be contaminated by external sources of DNA in the test environment and the DNA in the positive control. Use caution to avoid contamination of samples and reaction mix reagents.

The *therascreen* KRAS RGQ PCR Kit is not to be used for diagnosis of any disease.

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 DSP DNA FFPE Tissue Kit. Only the Rotor-Gene Q MDx instrument has been validated for use with the *therascreen* KRAS RGQ PCR Kit.

Performance Characteristics for CRC Specimens

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 8 FFPE human cell lines of which 7 harbor known KRAS mutations detected by the assay, and 1 KRAS wild-type (*i.e.*, no mutations at codon 12 and 13). Mutation status of samples was confirmed by bi-directional Sanger sequencing.

Cut-off

To establish the cut-offs for the assay, 220 FFPE samples were tested using a method following guidance in NCCLS EP17-A (20). The control reaction C_T range was established as 21.92 to 32.00. The cut-off 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 4.

Table 4. Established cut-off values for each mutation assay

	Mutant assay						
	12ALA	**12ASP**	**12ARG**	**12CYS**	**12SER**	**12VAL**	**13ASP**
Cut-off ($\leq\Delta C_T$)	8.0	6.6	8.0	8.0	8.0	7.5	7.5

Limit of blank

To assess performance of the *therascreen* KRAS RGQ PCR Kit in the absence of mutant positive template, and to ensure that a blank sample does not generate an analytical signal that may indicate a low concentration of mutation, samples with

no template were evaluated. The results demonstrated no detectable control or mutant C_T values in any of the mutation or control reaction tubes (internal control C_T values were all valid).

Comparison to analytical reference method

Two studies were conducted to demonstrate the concordance in mutation status of CRC samples tested with the *therascreen* KRAS RGQ PCR Kit relative to bidirectional sequencing. In the first study, 350 procured tumor samples from CRC patients were selected based on a set of baseline clinical, demographic and tumor specimen characteristics. Using a statistical random sampling technique, 150 samples of unknown mutation status were chosen for evaluation. These (150) FFPE samples were tested and subsequently analyzed in this study using statistical measures of agreement/disagreement from the CLSI EP12-A2 Guidance (21). A total of 137 of the FFPE samples returned valid results for both the *therascreen* KRAS RGQ PCR Kit and bidirectional sequencing.

The results demonstrated that the *therascreen* KRAS RGQ PCR Kit reported 2 samples as negative, 1 of which bidirectional sequencing indicated to be positive for 12ASP and the other indicated by bidirectional sequencing to be 13ASP. In contrast, 3 samples were reported as having a KRAS mutation by the *therascreen* KRAS RGQ PCR Kit which were not reported as positive by bidirectional sequencing. In addition, 1 sample identified as 12ARG by the *therascreen* KRAS RGQ PCR Kit was determined to be 12ASP by bidirectional sequencing. There were 5 samples determined to be mutation negative by bidirectional sequencing that were either indeterminate (3 samples) or invalid (2 samples) by the *therascreen* KRAS RGQ PCR Kit (data not shown). One sample that was indeterminate by Sanger bidirectional sequencing was determined to be 12SER by the *therascreen* KRAS RGQ PCR Kit (data not shown).

The results demonstrate a positive percentage agreement (PPA) of 96.3%, a negative percentage agreement (NPA) of 96.3%, and an overall percentage

agreement (OPA) of 96.4%. The overall results, excluding 6 failed Sanger samples, are shown with the respective 95% confidence interval (CI) in Table 5.

Table 5. Analysis of agreement

Measurement of agreement	Frequency (%)	95% 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

Table 6 (next page) shows details of the agreement between the *therascreen* KRAS RGQ PCR Kit and bidirectional sequencing.

Table 6. therascreen KRAS RGQ PCR Kit vs. Sanger bidirectional sequencing

KRAS Kit call	Mutation call by bidirectional sequencing								
	Negative	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP	Total
Negative	80	–	–	1	–	–	–	1	82
12ALA	–	3	–	–	–	–	–	–	3
12ARG	–	–	–	1	–	–	–	–	1
12ASP	–	–	–	20	–	–	–	–	20
12CYS	–	–	–	–	3	–	–	–	3
12SER	–	–	–	–	–	–	–	–	0
12VAL	2	–	–	–	–	–	14	–	16
13ASP	1	–	–	–	–	–	–	11	12
Total	83	3	0	22	3	0	14	12	137

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 bidirectional sequencing as described above.

A total of 13 (~5%) samples required macrodissection for *therascreen* KRAS RGQ PCR Kit evaluation. Out of the 271 samples with bidirectional sequencing results, 24 of the samples were indeterminate by the *therascreen* KRAS RGQ PCR Kit (failed control C_T range; data not shown). Concordance analysis was carried out on 247 samples with both valid bidirectional and *therascreen* KRAS RGQ PCR Kit results. There were 9 discordant samples. One sample from the 247 samples had a mutation positive result with bidirectional sequencing but a "No Mutation Detected" result with the *therascreen* KRAS RGQ PCR Kit. Eight samples were shown to have a positive result with the *therascreen* KRAS RGQ PCR Kit but a negative result with bidirectional sequencing. Overall, agreement was 96.4%. The data supports the accurate performance of the *therascreen* KRAS RGQ PCR Kit (Table 7 and Table 8).

Table 7. Analysis of agreement (second study)

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

Table 8 (next page) shows details of the agreement between the *therascreen* KRAS RGQ PCR Kit and bidirectional sequencing in the second study.

Table 8. therascreen KRAS RGQ PCR Kit vs. Sanger bidirectional sequencing (second study)

KRAS Kit call	Mutation call by bidirectional sequencing								
	Negative	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP	Total
Negative	132	–	–	–	–	1	–	–	133
12ALA	–	10	–	–	–	–	–	–	10
12ARG	5	–	5	–	–	–	–	–	10
12ASP	–	–	–	31	–	–	–	–	31
12CYS	1	–	–	–	11	–	–	–	12
12SER	–	–	–	–	–	13	–	–	13
12VAL	2	–	–	–	–	–	25	–	27
13ASP	–	–	–	–	–	–	–	11	11
Total	140	10	5	31	11	14	25	11	247

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 prespecified 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 cut-off ΔC_T value.

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. Eight FFPE cell lines; 7 with known mutant DNA content and 1 wild-type were used for this evaluation.

The proportion of mutant in total amplifiable DNA (percentage mutant DNA) was determined previously using a bidirectional Sanger sequencing method from unfixed cells, followed by relative peak analysis. In the case of 3 cell lines, the mutant content was 100% (i.e., the cell line DNA was homozygous mutant). The other cell lines were of mixed zygosity. Multiple DNA extractions from each sample were pooled to generate DNA stocks. The DNA stocks were then normalized to achieve target control reaction C_T values. Normalized mutant DNA extracts were diluted with normalized wild-type DNA extract to create a dilution series of extracts containing the same level of total amplifiable DNA but different levels of mutant DNA. Serial dilutions were then generated from these samples and run in multiple replicates.

The first dilution series was created for the mid-range control reaction C_T value (approximately 26). Nine replicates per dilution were tested. The percentage of correct calls as a function of the dilution for each mutant reaction is shown in Table

9. Highlighted boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Table 9. Percentage of correct calls as a function of the dilution for each mutation reaction

Mutation dilution (%)	Correct calls (%)						
	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
0.78	100	0	33.3	55.6	22.2	66.7	0
1.56	100	33.3	100	100	88.9	100	0
3.13	100	77.8	100	100	100	100	66.7
6.25	100	100	100	100	100	100	100
12.5	100	100	100	100	100	100	100
25.0	100	100	100	100	100	100	100
50.0	100*	100	100	100	100	100	100

* Mutation dilution for this sample was 40.0%.

The results of the first dilution series were used to generate dilutions for the confirmation of LOD values using narrower, reaction-specific ranges of percentage mutation dilutions at both low and high levels within the input range of the assay. For the high dilution series, 12 replicates of each were evaluated. The percentage of correct calls as a function of dilution for the high dilution series (target values approximately C_T 23–24) is shown in Table 10. Highlighted boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Table 10. Percentage of correct calls as a function of dilution for the high dilution series

Mutation	Mutation dilution high (%)					
12ALA	0.13	0.27	0.54	**1.08**	2.15	4.30
Correct calls (%)	0	0	91.7	**100**	100	100
12ASP	0.56	1.13	2.25	4.50	**9.00**	18.00*
Correct calls (%)	0	8.3	3.33	83.3	**100**	100
12ARG	0.16	0.33	0.65	**1.30**	2.60	5.20
Correct calls (%)	0	0	8.3	**100**	100	100
12CYS	0.12	0.24	0.49	0.98	**1.95**	3.90
Correct calls (%)	0	0	8.3	83.3	**100**	100
12SER	0.31	0.63	1.25	2.50	**5.00**	10.00
Correct calls (%)	0	0	33.3	66.7	**100**	100
12VAL	0.17	0.34	0.69	**1.38**	2.75	5.50
Correct calls (%)	0	0	16.7	**100**	100	100
13ASP	0.63	1.25	2.50	**5.0**	10.0	20.0
Correct calls (%)	0	0	0	**100**	100	100

* The number of valid replicates was 11.

For the low series, 24 replicates were evaluated per dilution, except where indicated. The percentage of correct calls as a function of dilution for the low dilution series (target approximately C_T value 31) is shown in Table 11. Highlighted boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Table 11. Percentage of correct calls as a function of dilution for the low dilution series

Mutation	Mutation dilution high (%)						
12ALA	0.27	0.54	1.08	2.15	**4.30**	8.60	12.90
Correct calls (%)	12.5	20.8	33.3	83.3	**100**	100	100
12ASP	0.56	1.13	2.25	4.50	**9.00**	18.0	27.0
Correct calls (%)	0	8.3	3.33	83.3	**100**	100	100
12ARG	0.33	0.65	1.30	2.60*	**5.20**	10.40	15.60
Correct calls (%)	8.3	4.2	29.2	52.2	**95.8**	100	100
12CYS	0.24	0.49	0.98	1.95	3.90	**7.80**	11.70
Correct calls (%)	8.3	4.2	20.9	54.2	83.3	**100**	100
12SER	0.63	1.25	2.50	5.00	10.0	20.0	**30.0**
Correct calls (%)	0	0	8.3	33.3	70.9	83.3	**100**
12VAL[†]	0.34	0.69	1.38	2.75	**5.50**	11.0	16.50
Correct calls (%)	4.3	16.7	46.7	75.0	**100**	100	100
13ASP	0.63	1.25	2.50	5.0	10.0	**20.0**	30.0
Correct calls (%)	0	4.2	8.3	33.3	70.8	**100**	100

* The number of valid replicates for 12ARG at 2.60 dilution was 23.

[†] The numbers of valid replicates in the series were 23, 24, 15, 16, 13, 12 and 19 for 12VAL.

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 which gave a predicted probability of detection of 0.95. The LODs determined from the dilution series beginning with either the low or high C_T values are shown in Table 12.

Table 12. Logistics regression data (LODs) for low and high C_T dilutions series

Assay	Low	High
12ALA	4.25	0.56
12ASP	10.23	6.43
12ARG	7.27	0.87
12CYS	6.90	1.21
12SER	25.75	4.20
12VAL	5.17	0.90
13ASP	18.83	4.16

The final LOD claims using FFPE cell lines when the input C_T value is between approximately 22 and 27 C_T are shown in Table 13. At the lower end of the C_T input range, the sensitivity of the assay decreases. The amount of input DNA may not contain sufficient copies to support the same percentage ratios of wild-type to mutant DNA observed within the high- and mid-points of the working range.

Table 13. LOD values for each mutations using FFPE cell lines

Assay	LOD C_{95} (percentage of mutant DNA in wild-type DNA)
12ALA	0.8
12ASP	6.4
12ARG	2.6
12CYS	1.5
12SER	5.6
12VAL	1.6
13ASP	6.4

Effect of input DNA

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 C_T) 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 14. For each of the mutations detected by the *therascreen* KRAS RGQ PCR Kit, the ΔC_T values measured at different total DNA input levels spanning the working range of the assay passed the pre-set acceptance criteria for the study. Although there is a slight increase in ΔC_T as DNA input increases, overall, the ΔC_T values were consistent across the working range of the *therascreen* KRAS RGQ PCR Kit within the prespecified acceptance criteria.

Table 14. Effect of DNA input on ΔC_T values across the input control reaction C_T range – FFPE cell lines

Mutation	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
12CYS	0.29	0.25	0.15	0.26	–0.1

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

Table continued from previous page

Table 14. Effect of DNA input on ΔC_T values across the input control reaction C_T range – FFPE cell lines (cont'd).

Mutation	Dilution 1 ~22–23 C_T	Dilution 2 ~24–25 C_T	Dilution 3 ~27–28 C_T	Dilution 4 ~29–30 C_T	Dilution 5 ~32–33 C_T
12SER	2.91	2.21	2.15	2.15	2.08
12VAL	0.98	0.71	0.58	0.81	0.67
13ASP	3.57	2.84	2.54	2.46	2.62

Linearity/amplification efficiency as a function of DNA input

The linearity and amplification efficiency of PCR for each mutation reaction, relative to the control reaction, across the working range of the *therascreen* KRAS RGQ PCR Kit was demonstrated. Amplification efficiency was calculated for each of the mutation reactions and the control reaction as [2(–1/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. The largest difference in the amplification efficiencies between the control reaction and a mutant reaction was observed for the 13ASP assay with a mean difference in efficiencies of approximately 14.5%. A summary of the data is shown in Table 15.

Table 15. **Summary of the data on amplification efficiency (function of DNA input)**

Sample		Intercept	Intercept standard error	Calculated slope	Standard error (slope)	Lower two-sided 95% confidence limit	Upper two-sided 95% confidence limit	Amplification efficiency	Difference in amplification
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
	13ASP C$_T$	26.466	0.106	−0.909	0.017	−0.945	−0.873	**1.144**	

Linearity/amplification efficiency as a function of percent mutation

The objective of this study was to evaluate the effect of serially diluted mutant positive sample on amplification efficiency, across the working range of the *therascreen* KRAS RGQ PCR Kit, beginning with input levels of C_T at approximately 22–23 C_T. DNA extracts from FFPE cell lines 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 ~23 C_T. The stocks were diluted serially 2-fold each time using wild-type DNA to maintain the total wild-type DNA constant while varying the percentage mutant DNA in the template. Thus, each of the templates generated had the same absolute quantity and concentration of DNA but differing ratios of wild-type to mutant DNA.

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. The mutation reaction C_T values were plotted and the efficiencies were calculated; the control reaction C_T values were consistent over the dilution series of each mutation. For each sample where the control reaction C_T value fell within the specified range (21.92–32.00), ΔC_T values were calculated. A linear regression model was fitted with mutation reaction C_T versus $\log_2$ DNA input dilution. The slope and 95% confidence intervals were reported.

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 with the amplification efficiencies differing by less than ± 10%. A summary of the data is shown in Table 16.

Table 16. Summary of the data on amplification efficiency (function of percent mutation)

Sample	Intercept	Intercept standard error	Calculated slope	Standard error (slope)	Lower two-sided 95% confidence limit (slope)	Upper two-sided 95% confidence limit (slope)	Amplification efficiency
12ALA	23.540	0.025	–0.968	0.010	–0.989	–0.947	1.047
12ASP	24.804	0.054	–1.030	0.022	–1.075	–0.985	0.960
12ARG	24.226	0.028	–1.008	0.11	–1.031	–0.984	0.990
12CYS	24.354	0.027	–0.981	0.011	–1.003	–0.959	1.027
12SER	25.376	0.054	–0.892	0.022	–0.937	–0.847	1.174
12VAL	22.703	0.035	–1.021	0.014	–1.050	–0.992	0.972
13ASP	27.555	0.057	–0.810	0.023	–0.857	–0.763	1.353

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, by means of spiking experiments at two concentrations, on the ΔC_T values and mutation status of test samples. The concentrations are shown in Table 17.

Table 17. Amount of interfering substances tested in each assay

Interfering substance	Actual high amount [µl/200 µl eluate]	Actual low amount [µl/200 µl eluate]
Paraffin wax (in xylene)	2.00×10^{-4}	5.00×10^{-5}
Xylene	2.00×10^{-4}	5.00×10^{-5}
Ethanol	1.35×10^{-3}	3.38×10^{-4}
Buffer ATL	5.40×10^{-4}	1.35×10^{-4}
Proteinase K	1.32×10^{-5}	3.30×10^{-6}
Buffer AL	1.33×10^{-3}	3.33×10^{-5}
Wash Buffer AW1	0.50	1.25×10^{-1}
Wash Buffer AW2	5.00	1.25

None of the potentially interfering substances evaluated at the concentrations expected to be encountered in normal use impacts 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 (see

page 67), 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 bidirectional 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 potentially leading to false positive results between DNA samples using the *therascreen* KRAS RGQ PCR Kit. Potential sources of cross-contamination include:

● Sample extraction (e.g., scraping of slides)

● Pipetting of samples

● Closure ("capping") of sample tubes

● Contamination of kit reagents during use

● Loading of assay tubes onto the Rotor-Gene Q MDx instrument

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

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

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

Exclusivity/cross-reactivity

The *therascreen* KRAS RGQ PCR Kit is comprised of 8 separate reactions; one 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 of KRAS mutations which the reaction is not intended to detect is evaluated to demonstrate 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 to 32.00), the highest concentration of DNA input is based on having a control C_T value of approximately 22. FFPE clinical samples were used for this evaluation, as well as FFPE cell line DNA.

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

To address the amount of nonspecific amplification of wild-type DNA by reaction mixes designed to amplify specific mutations, 60 replicates of wild-type FFPE cell-line DNA or DNA extracted from CRC tumor tissue at the highest concentration of amplifiable DNA input level were evaluated using the *therascreen* KRAS RGQ PCR Kit. For DNA extracted from the FFPE cell line, the control C_T values were approximately 22–23. Control C_T values for three wild-type CRC samples were between 24 and 25. The results demonstrated that the ΔC_T values exceeded the established cut-offs. The mean and lowest ΔC_T values observed for each reaction is shown in Table 18.

Table 18. Lowest mean ΔC_T observed for wild-type samples in mutant reactions

Mutant reaction	Cut-off	Lowest ΔC_T observed	Sample 1 ΔC_T mean (lowest)	Sample 2 ΔC_T mean (lowest)	Sample 3 ΔC_T mean (lowest)
12ALA	8	12.76	18.00 (11.40)	18.62 (11.50)	20.03 (19.36)
12ASP	6.6	10.35	10.90 (9.62)	10.34 (8.84)	10.68 (9.01)
12ARG	8	14.26	20.33 (12.94)	20.02 (13.20)	20.03 (19.36)
12CYS	8	13.66	20.62 (17.38)	20.29 (19.62)	20.03 (19.36)
12SER	8	11.97	17.26 (11.14)	17.90 (11.42)	18.05 (10.44)
12VAL	7.5	11.81	14.87 (11.46)	16.27 (11.50)	18.68 (11.36)
13ASP	7.5	10.94	12.35 (9.08)	13.68 (10.69)	14.82 (9.97)

Nonspecific amplification/cross-reactivity/exclusivity: mutation-positive KRAS DNA

Mutant samples that have a high concentration of input DNA were tested against all reaction mixes by preparing DNA samples from each of the FFPE cell lines so that the control reaction C_T corresponded to approximately 23. Six 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 are presented in Table 19 and demonstrate that there is cross-reactivity between mutant reactions. The 12ALA mutation was amplified and generated ΔC_T values below the ΔC_T thresholds for the 12CYS, 12SER and 12VAL reactions. The 12VAL mutation was amplified and generated a ΔC_T value below the ΔC_T threshold for the 12ALA reaction.

In all cases the results demonstrate that the correct mutation was called with the matched mutation reaction (i.e., the smallest ΔC_T value was the correct mutation call). All other test cases were either not detected or outside the ΔC_T threshold.

Table 19. ΔC_T cross-reactivity between mutation reactions using FFPE cell line DNA at the high input range

Mutant DNA	Cut-off	Mutation reaction (ΔC_T)*						
		12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
12ALA	8	**1.42**	12.66	–	5.81	2.78	6.31	13.21
12ASP	6.6	12.56	**2.42**	–	–	13.44	11.21	13.55
12ARG	8	13.12	11.56	**1.12**	11.42	–	13.43	12.66
12CYS	8	14.2	12.48	9.23	**0.98**	–	7.96	12.88
12SER	8	–	13.39	13.31	–	**3.02**	12.99	13.97
12VAL	7.5	6.83	–	–	–	13.38	**0.28**	13.74
13ASP	7.5	–	13.29	13.89	–	–	14.36	**4.5**

* ΔC_T values from matched reactions are shown in bold. Blank cells show no cross-reaction. ΔC_T from cross-reactive reactions below the cut-off are highlighted.

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 20 and Table 21.

Table 20. Assay variance estimates

	%CV for ΔC_T		%CV for mutant C_T		%CV for control C_T		
Assay	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 21. 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 22).

Table 22. Correct calls overall

	Correct calls	
Mutation	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

Sample handling variability

To assess sample handling variability as part of the *therascreen* KRAS RGQ PCR Kit process, 30 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. The variance of ΔC_T values for each assay was estimated, and the contribution of between and within laboratory sources was estimated using an ANOVA variance components model. Variance for within test sites was highest for 12ASP (0.30). Variance between test sites was highest for 12SER (0.05). A comparison by site of mean ΔC_T values with corresponding standard deviation (SD) for mutant and wild-type samples showed very close agreement for the results (Table 23 and Table 24). The results demonstrate the agreement of the DNA extraction procedure and sample processing in conjunction with the *therascreen* KRAS RGQ PCR Kit.

Table 23. Comparison by site of mean ΔC_T (SD) values for mutant type samples

	ΔC_T (Standard deviation)						
	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP
Site 1	2.44 (0.1)	2.62 (0.3)	3.03 (0.6)	2.24 (0.1)	2.34 (0.3)	2.51 (0.1)	3.93 (0.4)
Site 2	2.44 (0.2)	2.52 (0.4)	3.01 (0.7)	2.29 (0.2)	2.10 (0.4)	2.44 (0.5)	4.15 (0.7)
Site 3	2.67 (0.6)	2.52 (0.2)	3.07 (0.5)	2.29 (0.2)	2.74 (0.5)	2.56 (0.2)	3.95 (0.3)

Table 24. Comparison by site of mean ΔC_T (SD) values for wild-type samples

	ΔC_T (Standard deviation)						
	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP
Site 1	12.46 (0.3)	–*	10.37 (0.4)	–	11.84 (0.4)	12.36 (0.5)	11.11 (0.6)
Site 2	12.09 (0.6)	13.07 (0.2)	10.17 (0.5)	–	11.71 (0.7)	12.20 (0.6)	11.00 (0.9)
Site 3	12.07 (0.2)	–	10.61 (0.4)	–	11.94 (0.3)	12.28 (0.6)	11.82 (0.5)

* Denotes a missing value, due to no breakthrough being observed.

Lot interchangeability

The potential for lot-to-lot variability to impact the mutation detection was assessed. In this study, 3 lots of QIAamp DSP DNA FFPE Tissue Kit (FFPE extraction kit) and *therascreen* KRAS RGQ PCR Kit with each lot of FFPE extraction kit were evaluated. DNA was extracted from FFPE cell lines with 3 lots of FFPE extraction kit to yield DNA samples with target control C_T values of approximately 23, 26 and 31. These C_T values were selected to span the defined working range of total DNA input level for the *therascreen* KRAS RGQ PCR Kit (control C_T within 21.92–32.00). Six replicate extractions at each of the target C_T values were tested by each of the 3 independent *therascreen* KRAS RGQ PCR Kit lots. The ΔC_T values and mutation calls were collected for all test samples. The predefined study objectives were met with the correct mutation call observed in 100% of valid tests, confirming that sample mutation calling is not affected by using different lots of FFPE extraction kits and/or *therascreen* KRAS RGQ PCR Kits.

Performance Characteristics for NSCLC Specimens

Analytical performance

Limit of Detection (LoD)

The LOD values for the *therascreen* KRAS RGQ PCR Kit were previously determined using DNA extracted from FFPE Cell Lines. The objective for this study was to re-verify that the LOD of each of the mutation assays with NSCLC specimens is below or equal to 10% mutation content in the background of wild type DNA, when concentration of DNA is not a limiting factor. The LOD re-verification was performed using a mixture of Cell Line samples (12ALA, 12SER and 13ASP) and clinical FFPE NSCLC samples (12ASP, 12ARG, 12CYS and 12VAL). In a follow up study, clinical samples representing 12ALA, 12SER and 13ASP mutations were used. DNA extracted from mutation positive test samples, and wild type samples (to be used as diluents) were normalized to a medium DNA input level. The mutation positive samples were then diluted to low levels of mutant DNA content (defined as previously determined LOD). Mutation positive DNA samples were tested with the Control Reaction mix and relevant Mutation Reaction mixes. NSCLC samples diluted to the LOD mutation content level had an upper one-sided exact 95% confidence limit for the observed mutation positive detection rate greater than or equal to 95%, reverifying the CRC sample LOD values presented in Table 13.

Cell Correlation Study

The objective of this study was to determine the utility of FFPE NSCLC cell line samples as a suitable substitute for clinical FFPE NSCLC samples by comparing hit rates at, below, and above the established Limit of Detection (LoD) for DNA extracted from

FFPE cell line samples and DNA extracted from clinical FFPE NSCLC tissue samples. Furthermore, the study was performed to demonstrate that the FFPE cell line samples do not outperform clinical FFPE NSCLC samples when tested using the KRAS Kit.

The study used two KRAS mutation positive FFPE cell line samples (positive as detected by the KRAS Kit) and two *KRAS* clinical FFPE samples of the same mutation status. Six dilution levels that were expected to give C100 (1.5xLoD), C95 (LoD), C75, C50, C25 and C5 percent correct mutation calls were tested for each sample panel member. Two of the most prevalent KRAS codon 12 mutations G12C (12CYS) and G12D (12ASP) were used for this study.

Two Probit models were used for analysis. For the *KRAS* G12D mutation, the data outputs were not statistically significant indicating that there is no difference in performance between the cell line and clinical samples. However, for the KRAS G12C mutation, the positivity rate of the cell line was lower than that of the clinical samples at the different dilution levels, demonstrating that the cell line does not outperform the clinical sample. The study acceptance criteria were met whereby the 12ASP and 12CYS cell lines did not outperform the corresponding clinical samples and this the cell lines were used as substitutes where clinical samples were not available for validation studies.

Comparison to the Analytical Reference Method

The purpose of this study was to demonstrate the concordance in overall mutation status of the *therascreen*® KRAS RGQ PCR Kit NSCLC relative to bi-directional Sanger sequencing.

Table 25. Correlation Between Sanger and KRAS RGQ Result

Sanger status	KRAS Status		
Frequency (percent)	Mutation positive	No mutation detected	Total
Mutation positive	79	1	80
	(21.94	(0.28)	(22.22)
No mutation detected	21	259	280
	(5.83)	(71.94)	(77.78)
Total	100	260	360
	(27.78)	(72.22)	(100.00)

A set of 360 procured clinical NSCLC FFPE samples representing a mix of samples acquired by resection, FNA and CNB were used to evaluate the concordance between the KRAS Kit and Sanger bi-directional sequencing. DNA was extracted from each sample and tested with the KRAS Kit and bi-directional Sanger sequencing. Of the 360 valid samples, 338 samples produced concordant results between the KRAS Kit and bi-directional sequencing. Two of these concordant samples had one mutation call for the KRAS Kit but a double mutation for the bi-directional Sanger sequencing (one of which was the same as the one call by the KRAS Kit; therefore, a concordant result was given to these samples.

As seen in Table 26, the PPA was 98.75% (94.21, 99.94), the NPA was 92.50% (89.38, 94.92), and the OPA was 93.89% (91.39, 95.83).

Table 26. Agreement for Samples with both Sanger and KRAS Kit valid Results

Measure of agreement	Frequencies	Percentage agreement	Exact binomial lower two-sided 90% confidence limit	Exact binomial upper two-sided 90% confidence limit
Percentage Overall Agreement	338/360	93.89	91.39	95.83
Percentage Positive Agreement	79/80	98.75	94.21	99.94
Percentage Negative Agreement	259/280	92.50	89.38	94.92

There were 20 discordant samples; 19 were WT for the bi-directional Sanger sequencing but gave a mutation result for the KRAS Kit. One sample was WT for the KRAS Kit but gave a mutation positive (13ASP) status for the bi-directional Sanger sequencing.

Equivalency of sample acquisition

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 (RES) 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.

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 27).

Table 27. Agreement between Sample Acquisitions Methods (Including samples valid with all 3 acquisition 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	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
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

Table continued from previous page
Table 27. Agreement between Sample Acquisition Methods (cont'd)

Comparison	Agreement	Frequencies	Percentage (%)	Lower two-sided Confidence Limit	**Upper two-sided 95% Confidence Limits**
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
RES Vs. CNB with RES 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
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.

Repeatability and reproducibility

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 28 for each test panel and the quantitative precision values are presented in Table 29.

Table 28. 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 29. 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 29. 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 29. 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 29. 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 30. The quantitative precision values are presented in Table 31. The total reproducibility of the KRAS kit is shown in the #Total (SD, CV%) column of Table 31.

Table 30. 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	120/120	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 31. 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$_i$	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 on next page

Table 31. 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 31. 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$_i$	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

Table continued from previous page
Table 31. 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%)

Analytical accuracy (using clinical samples)

A study demonstrating the concordance in mutation detection of the therascreen KRAS RGQ PCR Kit relative to a validated Droplet Digital PCR (ddPCR) method was performed. A total of 319 samples with both ddPCR and *therascreen* KRAS RGQ PCR Kit results were analyzed to assess the positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA).

The 319 samples used in the analysis were a combination of clinical samples from Amgen Clinical Study 20170543 and procured NSCLC samples. For both methods, samples returning a result other than G12C were considered as mutation negative for this analysis. Of the 319 samples, 5 were invalid by ddPCR.

The OPA, NPA, and PPA, together with the corresponding two-sided 95% confidence intervals (CI), are summarized in Table 32. The OPA was 97.45%. A breakdown of the results is summarized in Table 33.

Table 32. OPA, PPA, and NPA of KRAS kit vs. ddPCR (two-sided 95% confidence interval)

Grouping variables	Proportion		Two-sided 95% confidence limit	
Group	Fraction	Percentage	Lower	Upper
Overall Percentage agreement (OPA)	306 / 314	97.45%	95.04%	98.89%
Positive percent agreement (PPA)	149 / 150	99.33%	96.34%	99.98%
Negative percent agreement (NPA)	157 / 164	95.73%	91.40%	98.27%

Table 33. 2x2 Frequency table KRAS kit vs. ddPCR

therascreen KRAS G12C result	ddPCR G12C result			
Frequency	G12C	G12C Not Detected	Invalid	Total
G12C	149	7	4	160
G12C Not Detected	1	157	1	159
Total	150	164	5	319

Non-specific amplification

The objective of this study was to assess the level of background amplification in wild type samples that have the highest concentration of input DNA within the KRAS kit working range. At high DNA input levels, the ΔC_T values observed from non-specific amplification in all 60 replicates of the clinical wild-type were above the reaction mix cut-off values. The *therascreen* KRAS RGQ PCR Kit correctly identified all 60 wild-type replicates as No Mutation Detected (wild-type).

Effect of input DNA

The DNA input level is the total quantity of amplifiable *KRAS* DNA (both mutant and wild type) in a sample. This is determined by the C_T value from the Control Reaction. When samples at different total DNA levels contain the same proportion of mutant DNA, it is expected that the measured ΔC_T values (*i.e.* the difference in C_T values between a Mutation Reaction and the Control Reaction) will remain consistent and therefore, that the sample mutation result will not be dependent upon, or influenced by, the DNA input level. This study included 2 parts.

Part 1 was designed to address the effect of input DNA levels on ΔC_T values. DNA extracted from nine FFPE NSCLC samples (4 Cell Lines and 5 Clinical) were used in this study. Samples were diluted to a Control Reaction C_T value of approximately 20.00-21.00C_T and from this, a pool of DNA for each mutation sufficient for 6 replicates was serially diluted 10-fold over 4 points to correspond to a C_T value of approximately 32.00–33.00C_T (Table 34).

Table 34. Dilution series for study on input DNA levels on ΔC_T values

Dilution factor	Initial pool	x10 (Dilution 1)	x100 (Dilution 2)	x1000 (Dilution 3)	x10000 (Dilution 4)
Expected Control C_T values	Approx. 20.00–21.00 C_T	Approx. 23.00–24.00 C_T	Approx. 26.00–27.00 C_T	Approx. 29.00–30.00 C_T	Approx. 32.00–33.00 C_T

This range was designed to span the DNA input range for samples (Control Reaction C_T of 21.92–32.00C_T), including one dilution point at a higher than acceptable DNA concentration and one dilution point at a lower than acceptable DNA concentration. All 5 DNA input levels for each mutation were tested using the Control reaction mix and corresponding Mutation reaction mix on a single PCR run.

Linear regression plots were generated to show the ΔC_T values for each mutation type across the dilutions within the Control C_T working range. The study acceptance criteria for part 1 of the study was met for all mutations as the linear regression lines and corresponding two-sided 95% confidence limits for the 3 dilutions within the Control C_T working range fell within the mean ± 1.96x Standard Deviation for Reproducibility as calculated in a repeatability and reproducibility study. The ΔC_T values are consistent across the working range of the *therascreen* KRAS RGQ PCR Kit for all assays, demonstrating that the DNA input level will not impact the mutation calling for samples.

Part 2 of the study was designed to evaluate the effect of serially diluting a mutant positive sample on amplification efficiency by testing samples with varying percentage mutation at a high DNA input level (Control Reaction of approximately 23.00C_T). DNA from clinical FFPE NSCLC and FFPE Cell Line sample pools was diluted to optical densities corresponding to Control Reaction C_T values of approximately 22.00-23.00C_T. Mutant DNA was serially diluted 2-fold into normalized wild-type DNA to create a series of 5 dilutions per mutant DNA sample that varied in their mutation percentage (targeting 100%, 50%, 25%, 12.5%, and 6.25% percentage mutant).

Table 35 summarizes all of the amplification efficiencies for each assay. Amplification efficiencies of wild-type DNA relative to mutant DNA present in a sample did not impact ΔC_T values and thus mutation call was not affected.

therascreen KRAS RGQ PCR Kit Instructions for Use (Handbook) 05/2021

Table 35. Amplification efficiencies of the mutation assay

Sample	Amplification efficiency
12ALA	0.921
12ASP	1.034
12ARG	0.916
12CYS	0.988
12SER	1.047
12VAL	1.082
13ASP	1.028*

* Value calculated with outlier excluded from analysis with outlier included amplification efficiency = 1.085

Sample handling variability

A study was performed to demonstrate that different laboratories produced the same results when analyzing the same clinical samples. This study used a combination of FFPE Cell Line and Clinical FFPE NSCLC samples. Thirteen Clinical FFPE NSCLC samples were used; three for 12ASP, three for 12CYS, and four for 12VAL mutations (a total of 10 mutation samples) plus three wild -type samples. Four FFPE Cell Line samples positive for the mutations 12ALA, 12ARG, 12SER, and 13ASP were used. Samples were blinded so that the mutation status was unknown to the operators at each of the three test sites. At each of the three sites, DNA extraction was performed on a batch of 20 FFPE sections (i.e. 10 pairs) per Mutation and wild-type. A total of 314 extractions were carried out across the three sites. All sample preparations at 3 individual test sites, when tested with the *therascreen* KRAS RGQ PCR Kit provided a correct mutation call for each of the seven mutations and the wild-type samples. The overall call for each of the seven mutations and the wild-type samples was 100% correct demonstrating inter-site consistency for the DNA extraction procedure and mutation detection using the KRAS 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.

Lot interchangeability

The objective of this study was to establish that the use of different lots of the QIAamp DSP DNA FFPE Tissue Kit for extraction and *therascreen* KRAS RGQ PCR Kit for *KRAS* mutation analysis did not have an impact on the sample mutation status of a tested sample. Three lots of each kit were used to test FFPE NSCLC samples to establish that the use of different lots of the QIAamp DSP DNA FFPE Tissue Kit and the *therascreen* KRAS RGQ PCR Kit did not impact the sample mutation status of tested samples. Twenty-four (24) NSCLC samples, three each for the seven mutations detected by the *therascreen* KRAS RGQ PCR Kit plus three wild-type samples were used in the study. Each sample was extracted using three lots of the QIAamp DSP DNA FFPE Tissue Kit. All DNA extracts were tested with each of the three lots of *therascreen* KRAS RGQ PCR Kit within the same PCR run on the RGQ instrument.

The ΔC_T values (the difference in C_T values between a Mutation Reaction and the Control Reaction) and mutation calls were collected for all test samples extracted with different QIAamp DSP DNA FFPE Tissue Kits and tested with

different *therascreen* KRAS RGQ PCR Kits. The acceptance criterion of "at least 95% of mutations calls must be correct for the overall proportion" was met with each mutation and wild-type being called 100% correctly.

Stability of FFPE sections

The main objective of this study was to demonstrate that sections prepared from resected FFPE NSCLC samples were stable when stored in the dark at ambient temperature. Sample sections from five KRAS mutation positive clinical FFPE NSCLC samples (one of each of the mutations 12ALA, 12ASP, 12CYS, 12VAL, and 13ASP) and one KRAS wild-type sample were sectioned and stored until required. The sections were tested with the *therascreen* KRAS RGQ PCR Kit over five time points spanning 75 weeks. All samples provided the correct mutation status call at all the time points. This study demonstrated that FFPE sections are stable at ambient temperature for at least 70 weeks.

A second study was performed to demonstrate that sections prepared from FFPE NSCLC FNA samples are stable when stored in the dark at ambient temperature.

This study used FNA samples that are wild type for the seven KRAS mutations that are detected by the *therascreen* KRAS RGQ PCR Kit. Sections from ten KRAS wild-type samples were used. These samples were extracted using one lot of the QIAamp DSP DNA FFPE Tissue Kit. DNA was extracted and tested at T0, then at T1 (T0+52 weeks), T2 and T3, which spanned an additional eight weeks after T1. The results obtained during this study provide evidence that FFPE NSCLC FNA samples are stable for at least 56 weeks when stored in the correct conditions.

Interfering substances

This study was designed to demonstrate that the presence of a potentially interfering substance would not produce any false positive or false negative results when testing was performed using the *therascreen* KRAS RGQ PCR Kit. Eight (8) potentially

interfering substances from the DNA extraction process were identified: paraffin wax, xylene, ethanol, Buffer ATL, Proteinase K, Buffer AL, Buffer AW1, and Buffer AW2. Each substance was tested using DNA extracted from eight FFPE Cell Lines, representing each of the seven mutations detected by the *therascreen* KRAS RGQ PCR Kit, and a wild type sample. The mutation samples were tested at a level corresponding to approximately three times the limit of detection (3xLoD). Both analytes (mutation and wild-type samples) were spiked with each of the eight potential interfering substances at three different levels: the highest expected carry over (1x), ten-times the highest expected carry over (10x) and one hundred-times the highest expected carry over (100x). To act as a negative control, both analytes were also spiked with Buffer ATE, the final elution buffer in the DNA extraction process. The study demonstrated that the substances identified from the FFPE DNA extraction kit which might carry over into the *therascreen* KRAS RGQ PCR Kit did not have any adverse effect on the performance of the assay at the 1x level of interferent; the correct mutation call was always given and the presence of the interfering substance did not have a statistically significant effect on difference in ΔC_T means for the majority of sample conditions tested (58 out of 64 conditions, at 1x level). For the six samples that did show a statistically significant difference, the observed difference in the means for each sample was within the study acceptance criterion of ±2xSD (SD estimate taken from a reproducibility study).

An additional study was performed to assess the impact of Hemoglobin (Hb) on the *therascreen* KRAS RGQ PCR Kit. In this study, procured clinical resection samples for the 12ALA, 12ASP, 12ARG, 12VAL, 12CYS, and 13ASP mutations were used. A 12SER clinical sample was not assessed due to the rarity of the mutation. A clinical *KRAS* wild type FFPE sample was incorporated into the study sample set to assess any potential interference in samples with a mutation negative status. Test samples were spiked with Hb at two different concentrations; half the highest expected carry over (0.5x) and the highest expected carry over (1x). The recommended test concentration (1x) was taken from CLSI EP07-A2 as 2g/L. This study demonstrated that the detection of the 12ASP, 12ARG, 12VAL, 12CYS, and 13ASP mutations by the

therascreen KRAS RGQ PCR Kit is not impacted by the potential interferant Hemoglobin. Detection of the 12ALA mutations by the KRAS kit however is impacted at a concentration of 0.50 µg/µl Hb or higher.

Guardband Studies

The objective of the guardbanding studies was to establish the robustness of the KRAS Kit. Changes were assessed for any potential effects on mutation reporting. The following studies were conducted to assess the robustness of the KRAS Kit:

Varying Proteinase K Digestion Times

The objective of this study was to provide guardband study data demonstrating the robustness of the *therascreen* KRAS RGQ PCR System to varying proteinase K digestion times (50, 55, 60, 65 and 70 minutes). Eight non-small cell lung cancer (NSCLC) samples, representing each of the seven mutations detected by the KRAS Kit along with one wild type (WT) (four FFPE cell lines and four clinical FFPE NSCLC samples), were extracted using the FFPE Extraction Kit and tested (6 replicates) using one lot of the KRAS Kit NSCLC. The DNA input level of the samples was determined using the KRAS Kit Control Reaction mix. Mutant samples were tested using the Control Reaction mix and relevant Mutation Reaction mix; WT samples were tested using all eight reactions of the KRAS Kit. The ΔC_T values (the difference in FAM C_T values between a Mutation Reaction and the Control Reaction) and mutation calls for all samples were obtained at each digestion time point.

At the different Proteinase K digestion time points of 55, 60, and 65 minutes, all samples were called with 100% correct mutation calls, demonstrating the robustness of the KRAS System. Overall, varying Proteinase K digestion times (50 min – 70min) had no significant effect upon the performance of the KRAS NSCLC system. For the entire study, 238/239 valid samples gave the correct mutation call with a single valid WT replicate giving a 12ASP mutation result at 70 minutes. Since the five other replicates for this sample all gave the expected WT call, no investigation was initiated.

An additional study was also performed assessing the 12ARG mutation (using a human lung cell line FFPE sample) in order to address the deficiencies in the initial study where a pancreatic cell line was used to represent the 12ARG mutation. An FFPE cell line sample which represented the 12ARG mutation detected by the KRAS Kit, was tested in this study. Similar to the study described above, the samples processed for 55, 60, 65 and 70 minutes were assessed. All reaction replicates (144) yielded the correct call.

Guardband Study on Volumetric Tolerance

The objective of this study was to determine the tolerance of the *therascreen* KRAS Kit to variations in pipetting volumes. Cell lines were used for these studies. This study used eight FFPE cell line samples (12ALA, 12ASP, 12ARG, 12CYS, 12SER, 12VAL, 13ASP and WT), Positive Control (PC) and No template Control (NTC). The volume of each PCR component (i.e., each reaction mix, sample or *Taq* polymerase) of the KRAS Kit was varied individually and incrementally up to approximately ±20% while all other components were used at the standard volumes for the kit. Each variable was considered to be a test condition (TC). Each run was set up in the standard manner, with the exception of the pipetting volumes. The volumes of reagents used for each run were varied so that in total 23 test conditions were analyzed. This guardband study demonstrated that the *therascree*n KRAS RGQ PCR System is robust up to approximately ±20% in varying pipette volumes for all assays other than 12ASP. When the volume of each PCR component of the KRAS Kit was varied individually and incrementally while all other components were used at the standard volumes, there was no change in mutation call and all differences in mean ΔC_T were within ±2 x the intermediate precision observed in Repeatability and Reproducibility for all assays, other than 12ASP. The result obtained with the 12ASP assay with the volumetric tolerance level for -21% *Taq* did not pass the acceptance criteria. The 12ASP sample met the acceptance criteria for ±8% volumetric tolerance; therefore, this level was set for this reaction.

An additional guardband study was also performed assessing the 12ARG mutation (using a human lung cell line FFPE sample) in order to address the deficiencies in the initial study where a pancreatic cell line was used to represent the 12ARG mutation. An FFPE Cell Line sample which represented the 12ARG mutation detected by the KRAS Kit, was tested in this study. The sample was tested with all 23 test conditions, in triplicate. In order to recreate conditions most likely to result in a false positive or false negative result, the volumes of multiple components (Reaction Mix, *Taq*, and Sample) were altered simultaneously. The acceptance criterion for the study was that the differences in the mean delta C_T values between the different PCR component levels should either be not significantly different from zero or the estimate should be less than or equal to twice the intermediate precision value calculated during the Repeatability and Reproducibility study. All results fulfilled these requirements. This Guardband study demonstrated that the KRAS Kit is robust enough to tolerate volumetric reagent changes up to ±20% (when all other factors are constant) due to pipetting error.

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 issue 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 companion diagnostic test 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 36 and Figure 1.

Table 36. 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		–		–	
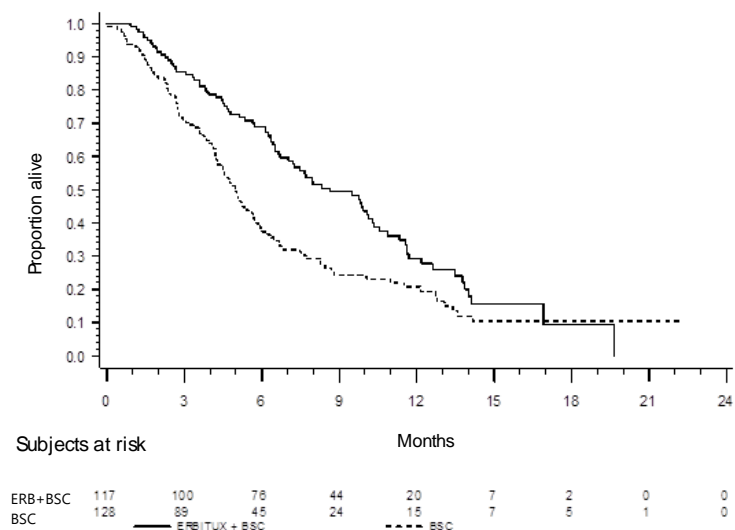
Figure 1. **Kaplan-Meier curve for overall survival in patients with KRAS mutation-negative (wild-type) metastatic colorectal cancer.**

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 companion diagnostic test 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 37 and Figure 2.

Table 37. 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%)
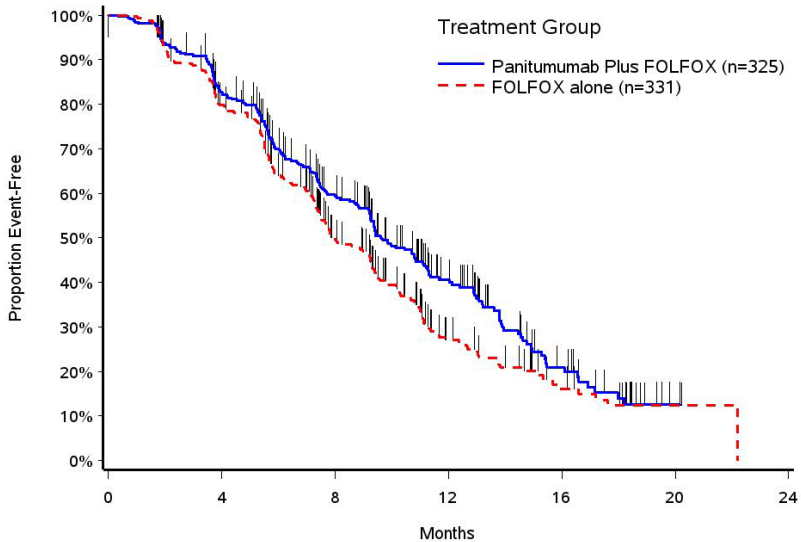
Figure 2. **Kaplan-Meier plot of progression-free survival (PFS) in patients with wild-type KRAS mCRC.**

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 3.
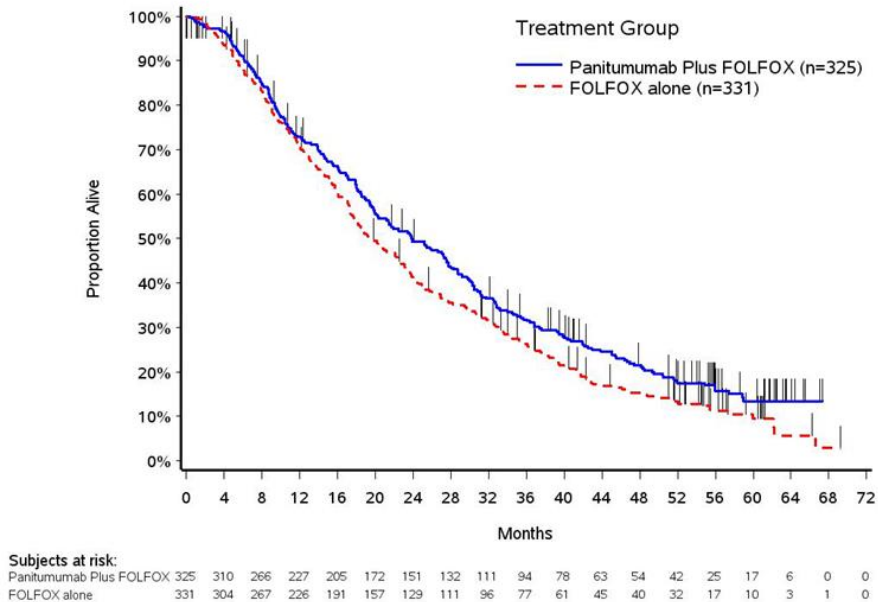
Figure 3. **Kaplan-Meier Plot of overall survival (OS) in patients with Wild-type KRAS mCRC.**

Clinical study supporting use of LUMAKRAS™ (sotorasib)

A clinical performance study was conducted to demonstrate the clinical validity of the *therascreen* KRAS RGQ PCR Kit as a companion diagnostic test that aids in the identification of NSCLC patients for treatment with LUMAKRAS (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 LUMAKRAS (sotorasib) treatment. The clinical trial 20170543 is an ongoing open-label, multicenter, phase 1/2 study designed to evaluate the efficacy and safety of LUMAKRAS (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 companion diagnostic test. 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 LUMAKRAS (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 36% (45 of 124 subjects; 95% CI: 28-45%); two (2) subjects (1.6%) achieved complete response and 43 subjects (35.8%) achieved partial response.

For more information regarding Erbitux Vectibix and LUMAKRAS indications, refer to the most recent drug labels at
www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm.

References

1. Hilger, R.A., Scheulen, M.E., Strumberg, D. (2002) The Ras-Raf-MEK-ERK pathway in the treatment of cancer. Onkologie **25**, 511.

2. Bachireddy, P., Bendapudi, P.K., Felsher, D.W. (2005) Getting at MYC through RAS. Clin. Cancer Res. **11**, 4278.

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

4. Neumann, J., Zeindl-Eberhart, E., Kirchner, T., Jung, A. Epub 2009 Frequency and type of KRAS mutations in routine diagnostic analysis of metastatic colorectal cancer. Pathol. Res. Pract. **205**, 858.

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

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

7. Van Cutsem, E., et al. (2008) K-RAS status and efficacy in the first-line treatment of patients with metastatic colorectal cancer (mCRC) treated with FOLFIRI with or without cetuximab: The CRYSTAL experience. J. Clin. Oncol. **26**, (May 20 suppl; abstr 2).

8. Tejpar, S., et al. (2008) Relationship of efficacy with K-RAS status (wild type versus mutant) in patients with irinotecan-refractory metastatic colorectal cancer (mCRC), treated with irinotecan (q2w) and escalating doses of cetuximab (q1w): The EVEREST experience (preliminary data). J. Clin. Oncol. **26**, (May 20 suppl; abstr 4001).

9. Amado, R.G., et al. (2008) Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer: Results from a phase III trial of panitumumab compared to best supportive care. J. Clin Oncol. **26**,1626.

10. Freeman, D.J. et al. (2008) Association of K-ras mutational status and clinical outcomes in patients with metastatic colorectal cancer receiving panitumumab alone. Clin. Colorectal Cancer **7**,184.

11. Freeman, D., et al. (2007) Association of somatic KRAS gene mutations and clinical outcome in patients (pts) with metastatic colorectal cancer (mCRC) receiving panitumumab monotherapy. Eur. J. Cancer **5**, 239.

12. Douillard, J.Y., et al. (2010) Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: The PRIME study. J. Clin. Oncol. **28**, 4697.

13. Peeters, M., et al. (2010) Randomized phase III study of panitumumab with fluorouracil, leucovorin, and irinotecan (FOLFIRI) compared with FOLFIRI alone as second-line treatment in patients with metastatic colorectal cancer. J. Clin. Oncol. **28**, 4706.

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

15. Arbour, K.C., et al. (2018). Effects of Co-occurring Genomic Alterations on Outcomes in Patients with *KRAS*-Mutant Non-Small Cell Lung Cancer. Clin Cancer Res. **24**(2): 334-340.

16. Newton, C.R., et al. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. **17**, 2503.

17. Whitcombe, D. et al. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotech. **17**, 804.

18. Thelwell, N., et al. (2000) Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Res. **28**, 3752.

19. Catalog of Somatic Mutations in Cancer **www.sanger.ac.uk/genetics/CGP/cosmic**.

20. Clinical and Laboratory Standards Institute (CLSI) (2004). *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures: Approved Guideline*. CLSI Document EP17-A. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

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

22. Clinical and Laboratory Standards Institute (CLSI) (2004). *Evaluation of Precision Performance of Quantitative Measurement Methods: Approved Guideline*, 2nd ed. CLSI Document EP5-A2. Wayne, PA: Clinical and Laboratory Standards Institute (formerly NCCLS).

therascreen KRAS RGQ PCR Kit Instructions for Use (Handbook) 05/2021

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
IVD	In vitro diagnostic medical device
REF	Catalog number
(manufacturer symbol)	Manufacturer
MAT	Material number
Rn	R is for revision of the Instructions for Use (Handbook) and n is the revision number
LOT	Lot number
GTIN	Global Trade Item Number
(hourglass symbol)	Use by
(Σ triangle symbol) <24>	Contains reagents sufficient for 24 reactions
COMP	Components (*i.e.* a list of what is included)

Symbol	Symbol definition
CONT	Contains (contents)
NUM	Number (*i.e.* vials, bottles)
	Temperature limitation
	Consult instructions for use
	Keep away from sunlight

Appendix: Installation of the Rotor-Gene Q *therascreen* KRAS Assay Package

The *therascreen* KRAS RGQ PCR Kit is designed for use with the Rotor Gene Q MDx instrument with a 72-well rotor. The Rotor-Gene Q *therascreen* KRAS Assay Package is available separately on CD (QIAGEN, cat. no. 9023818).

Note: The Rotor-Gene Q *therascreen* KRAS Assay Package software will only work with Rotor-Gene Q software version 2.3. Make sure the correct version of Rotor-Gene Q software is installed before proceeding with the *therascreen* KRAS Assay Package installation.

The package includes "*therascreen* KRAS QC Locked Template" and "*therascreen* KRAS Locked Template." The CD also includes printable plate maps that can be used to record the experiments and run templates that define the PCR protocols for FFPE sample preparation using the QIAamp DSP DNA FFPE Tissue Kit, DNA sample assessment using the *therascreen* KRAS RGQ PCR Kit and detection of KRAS mutations using the *therascreen* KRAS RGQ PCR Kit.

Follow this procedure to install the *therascreen* KRAS Assay Package.

1. Order the Rotor-Gene Q *therascreen* KRAS RGQ Assay Package CD version 3.1.1 (QIAGEN, cat. no. 9023818) available separately from QIAGEN.
2. Insert the CD into the CD drive of the laptop connected to the Rotor-Gene Q MDx instrument.
3. Start the installation by double-clicking the file **therascreen_KRAS_Assay_Package_3.1.1.exe**.
   The setup wizard will appear.
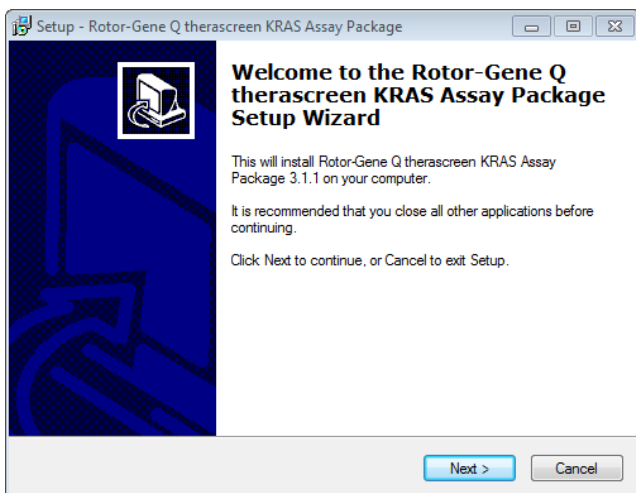
4. Click **Next** to continue.

   The **License Agreement** dialog box opens.
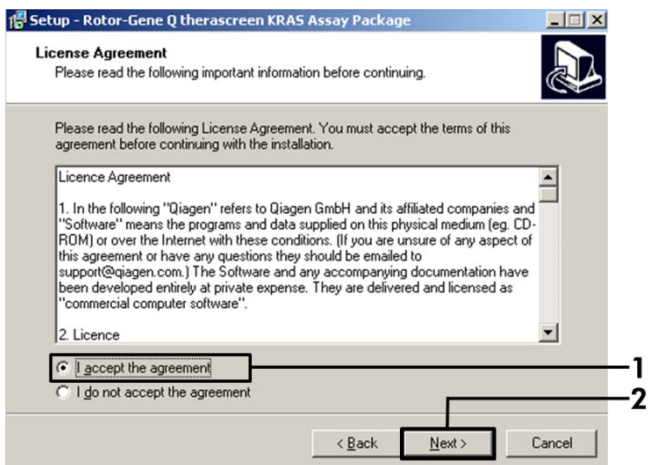
5. Read the License Agreement in the **License Agreement** dialog box and accept the agreement by checking the statement **I accept the agreement** (1).

6. Click **Next** (2) to continue.

   The template setup will start automatically and a final **Setup** dialog box will
   appear.
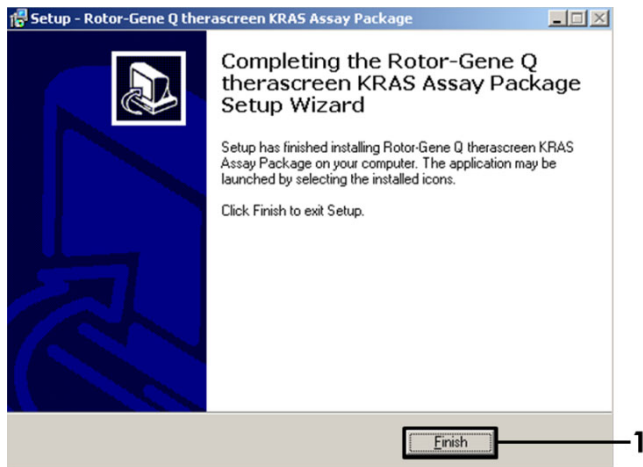
7. Click **Finish** (1) to exit the setup wizard

8. Restart the computer.

   Shortcuts to both the **therascreen KRAS QC Locked Template** and **therascreen
   KRAS Locked Template** will be generated automatically and 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	870021
Rotor-Gene Q *therascreen* KRAS Assay Package 3.1.1 CD	Software protocol package for use with the *therascreen* KRAS RGQ PCR Kit and the QIAGEN Rotor-Gene Q MDx instrument (compatible with Rotor-Gene Q software version 2.3)	9023818
Rotor-Gene Q MDx and accessories		
Rotor-Gene Q MDx Platform (US)	Real-time PCR cycler, laptop computer, software, accessories, 1-year warranty on parts and labor	9002035
Rotor-Gene Q MDx System (US)	Real-time PCR cycler, laptop computer, software, accessories, 1-year warranty on parts and labor, installation and training	9002036
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

Product	Contents	Cat. no.
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106
Related products		
QIAamp DSP DNA FFPE Tissue KRAS Kit (50)	For 50 DNA preps: QIAamp MinElute® Columns, Proteinase K, Buffers, and Collection Tubes (2 ml)	60404

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
R8, September 2017	Updated manufacturer (front cover).
R9, January 2020	Updated address of legal manufacturer (front cover)
	Interchanged 12ASP and 12ARG rows in Tables 15, 19, 20, and 21
	Interchanged 12ASP and 12ARG columns in Tables 22 and 23
	Minor formatting changes
R10, May 2021	Updated the Intended Use section to include the use of NSCLC tissue and LUMAKRAS™ (sotorasib)
	Updated the Summary and Explanation section to include information about NSCLC patients
	Revised Specimen and Handling Storage to differentiate instructions for preparing tissue specimens from CRC tissue and NSCLC tissue
	Added DNA extraction for NSCLC samples procedure
	Revised Table 13 to correct LOD values for 12ALA and 12ASP
	Added Performance Characteristics for NSCLC Specimens chapter
	Updated Clinical performance section to add topic about the clinical study supporting the use of LUMAKRAS