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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 instrument

For use with QIAamp[®] DSP DNA FFPE Tissue Kit

Version 1

IVD

REF

870021



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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 7 somatic mutations in the human KRAS oncogene using DNA extracted from formalin fixed paraffin-embedded (FFPE) colorectal cancer (CRC) tissue. The *therascreen* KRAS RGQ PCR Kit is intended to aid in the identification of CRC patients for treatment with Erbitux® (cetuximab) or with Vectibix® (panitumumab) based on a KRAS no mutation detected test result.

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 (4, 5–13). Such mutations in the KRAS oncogene are present in around 40% of cases (14).

Using Scorpions® (15) and ARMS (Allele Refractory Mutation System) technologies (16, 17), 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 1). Based on data in the COSMIC database (2012 v59), the seven mutations detected by the KRAS Kit account for >97% of all reported KRAS mutations in CRC patients (18).

Table 1. List of mutations and COSMIC identities*

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

* COSMIC IDs are taken from the Catalog of Somatic Mutations in Cancer: (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 principle components of the kit are explained below.

Mutation reaction mixes

Each mutation-specific reaction mix uses a mutation-specific 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 (HEXTM) in order to distinguish it from the FAM labeled Scorpions in the control and mutation reactions.

Positive control

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.

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- Refer to “Materials Required but Not Provided,” page 15, 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 95, for instructions on installing the Rotor-Gene Q *therascreen* KRAS Assay Package.

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

Run parameters

The Rotor-Gene Q MDx 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 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 buffer, and the positive control contains the carrier Poly A RNA.

Table 2. 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 μ l

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

Reagent	Ingredients	Quantity
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
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

Reagent	Ingredients	Quantity
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
<i>Taq</i> DNA Polymerase	<i>Taq</i> 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

<i>therascreen</i> 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	<i>Taq</i> 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
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 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

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- 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 23)

Warnings and Precautions

Warnings

For in vitro diagnostic use

For prescription use only

For use with Rotor-Gene Q MDx 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 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 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 ($2-8^{\circ}\text{C}$)
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 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.

To prepare tissue samples for DNA extraction:

- Using standard materials and methods, fix the tissue specimen in 10% neutral buffered formalin (NBF), and embed the tissue specimen in paraffin. Using a microtome, cut 5 μm serial sections from the paraffin block and mount them on glass slides.
- Use a trained individual (e.g., a pathologist) to assess a Hematoxylin & Eosin (H&E)-stained section for tumor content and 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 $\leq 20\%$ tumor content by area, macrodissect one or more sections. Discard the non-tumor tissue.
- For sections that are $< 4 \text{ mm}^2$ 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.

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- 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.

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.

Procedure

DNA extraction

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 samples 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 methyl ethyl ketone.

- 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.

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 software is installed before first use of the Rotor-Gene Q MDx instrument (see “Appendix: Installation of the Rotor-Gene Q *therascreen* KRAS Assay Package,” page 95).
 - 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 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 19). 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)*
<i>Taq</i> 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	-	-	-	-	-	-

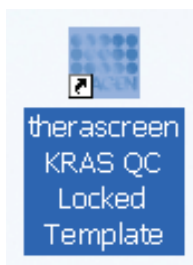
5. Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the control assay master mix by fully aspirating up and down 10 times.
6. Immediately add 20 µl control assay master mix to each PCR strip tube. Refer to the table for the tube layout.
For DNA sample assessment, control 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 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.

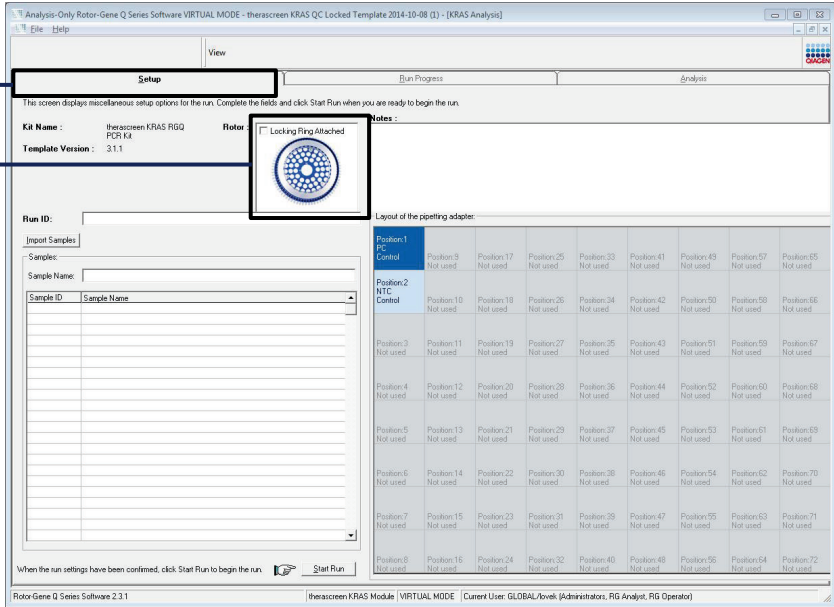
Note: If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor Gene Q MDx instrument is maintained.

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

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 instrument.

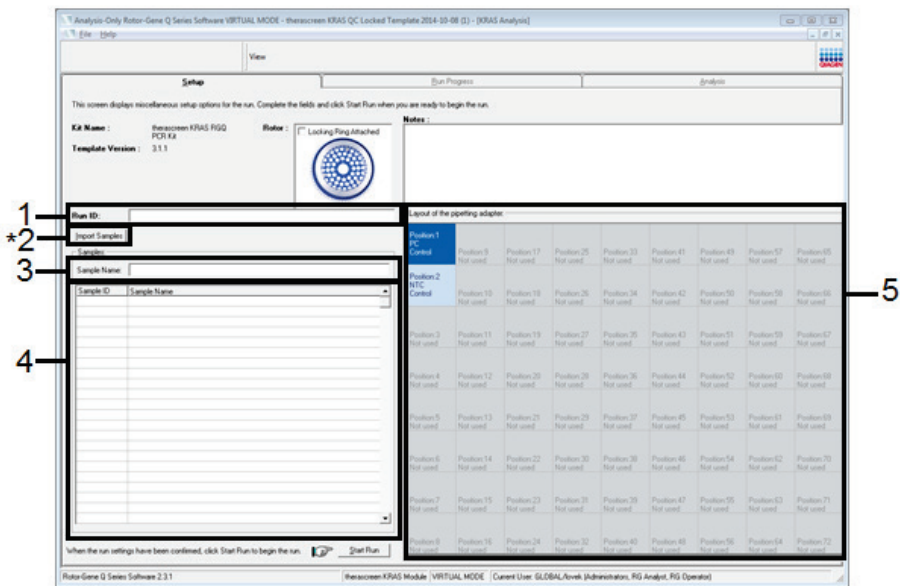


15. The **Setup** tab appears (1) as the default. Ensure that the locking ring is properly attached and check the **Locking Ring Attached** box (2). Close the lid of the Rotor-Gene Q MDx instrument.



1
2

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.

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.

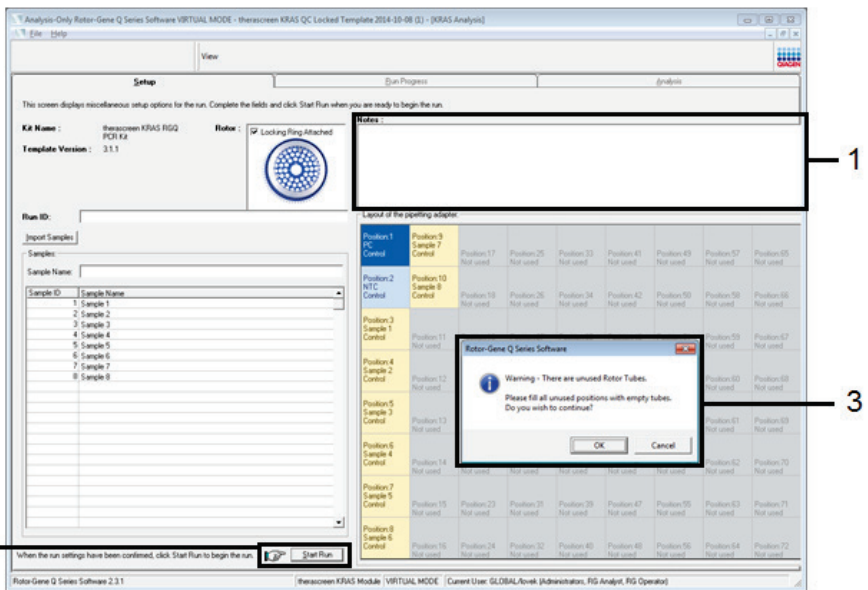
Note: In the **Layout of the pipetting adapter** panel, check that addition of the sample name has been highlighted by a change in color and the sample name is in the sample position.

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

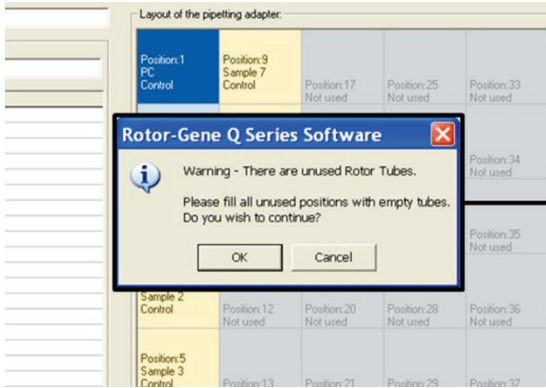
18. Repeat step 17 to enter the names of all additional samples.

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).

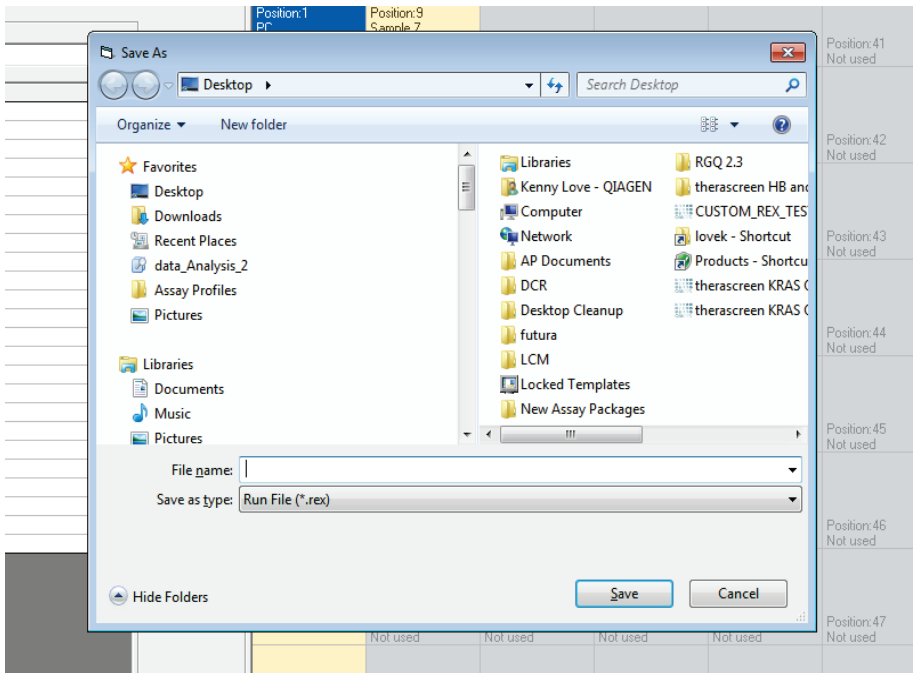
19. When all sample names have been entered, verify they are correct. Add any additional information in the **Notes** dialog field (1) if necessary and then click the **Start Run** button (2).



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.

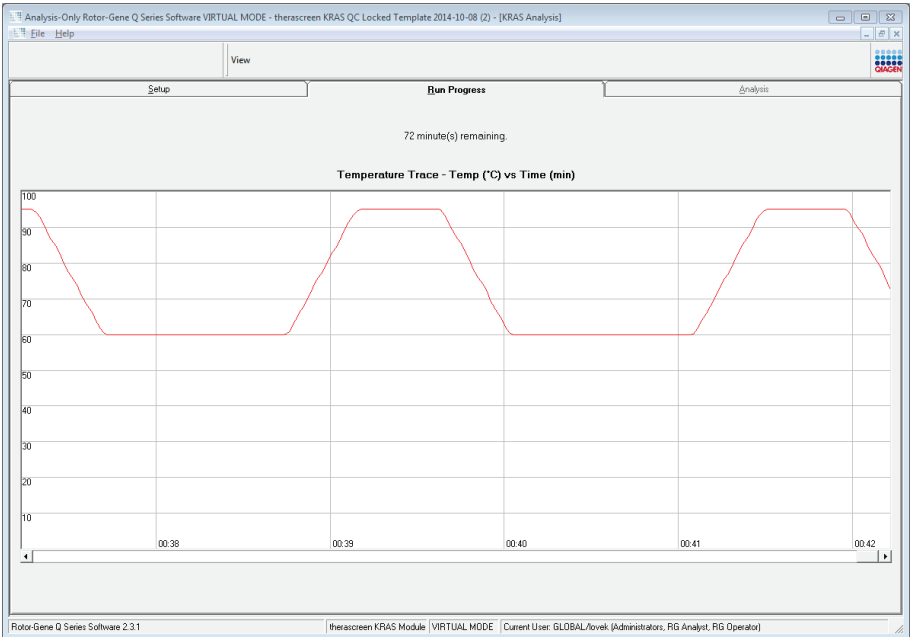


20.A **Save As** window appears. Chose an appropriate file name and save the PCR run as a *.rex run file to the selected location and click the **Save** button.



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.

Analysis-Only Rotor-Gene Q Series Software VIRTUAL MODE - thetascreen KRAS QC Locked Template 2014-10-08 (1) - [KRAS Analysis]

File Help

View

Setup Run Progress Analysis

Report

QC Sample Result Table:

Tube ID	Sample Name	Control Assay C _T	Flags/Warnings	Status
1	PC Control	27.40	-	Valid
2	NTC Control	-	-	Valid
3	Sample 1	27.40	-	Valid
4	Sample 2	27.63	-	Valid
5	Sample 3	29.36	-	Valid
6	Sample 4	29.36	-	Valid
7	Sample 5	30.94	-	Valid
8	Sample 6	31.16	-	Valid
9	Sample 7	27.63	-	Valid
10	Sample 8	29.36	-	Valid

Rotor-Gene Q Series Software 2.3.1 | thetascreen KRAS Module | VIRTUAL MODE | Current User: GLOBAL/lovek (Administrator, RIG Analyst, RIG Operator)

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 *thetascreen* KRAS Assay Package flags," page 50, and "Troubleshooting Guide," page 56).
- 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.

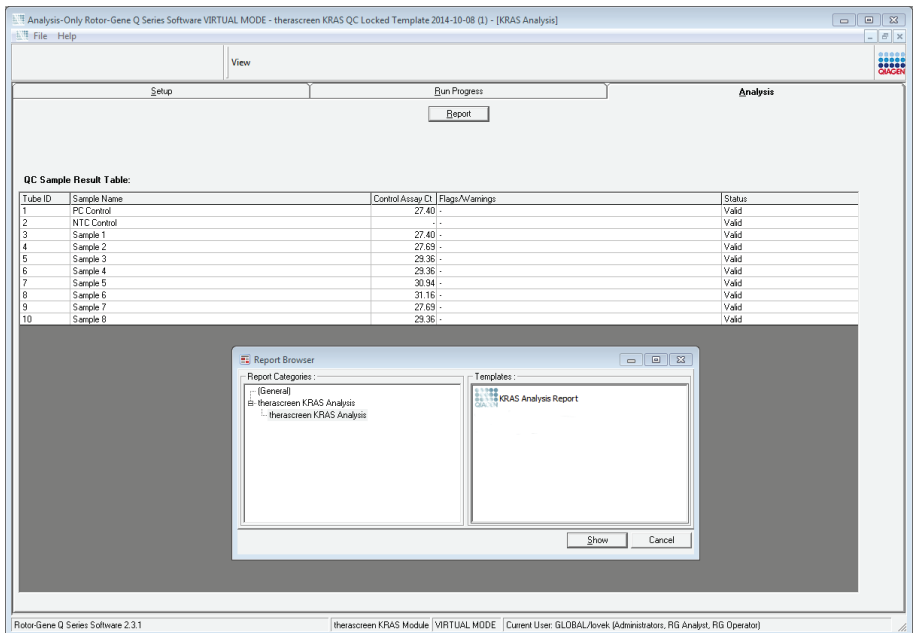
Dilute to a C_T of 21.92–32.00. A 1:1 dilution increases the C_T value by approximately 1.0.

- Sample control reaction C_T is 21.92–32.00, ($21.92 \leq \text{Control } C_T \leq 32.00$): 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 19). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

- 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.
- 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 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
12ALA (tube 12ALA)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
12ASP (tube 12ASP)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
12ARG (tube 12ARG)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
12CYS (tube 12CYS)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
12SER (tube 12SER)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
12VAL (tube 12VAL)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$
13ASP (tube 13ASP)	19.76 $\mu\text{l} \times (n + 1)$	0.24 $\mu\text{l} \times (n + 1)$

* 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.

- 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	Controls		Sample number						
	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

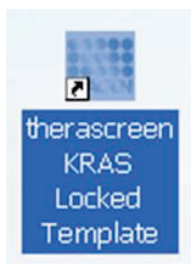
- Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the assay master mixes by fully aspirating up and down 10 times.
For detection of KRAS mutations, assay master mixes should be added to 8 PC tubes, 8 NTC tubes, and 8 tubes for each DNA sample.
- Immediately add 5 μ l of nuclease-free water for no template control (tube NTC) to the NTC tubes (tube positions 9–16) and cap the tubes.
- Add 5 μ l of each DNA sample to the sample tubes (tube positions 17–72) and cap the tubes.
- Add 5 μ l of KRAS Positive Control (tube PC) to the PC tubes (tube positions 1–8) and cap the tubes.
- 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.
- 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.

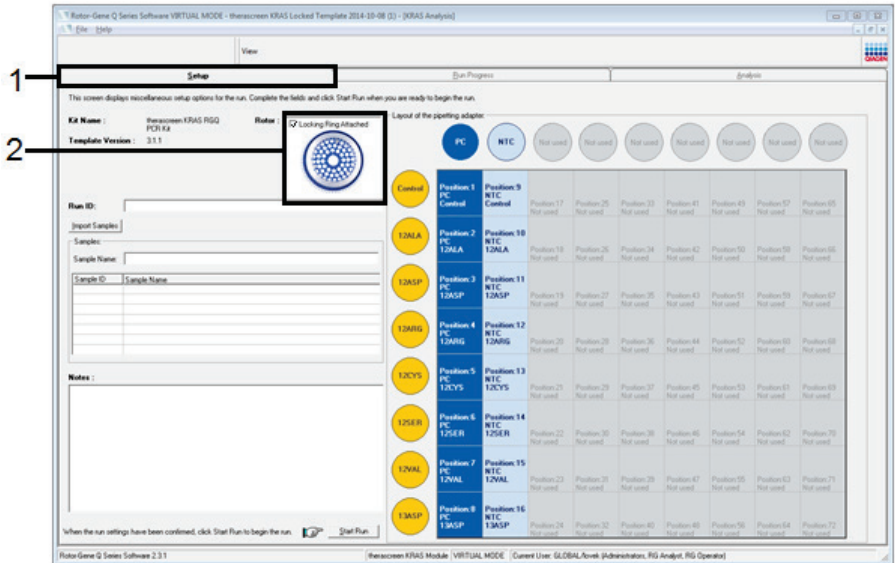
Note: A maximum of 7 samples can be included in each PCR run. If the rotor is not fully occupied, all unused positions on the rotor must be filled with a capped, empty tube. This ensures that the thermal efficiency of the Rotor-Gene Q MDx instrument is maintained.

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

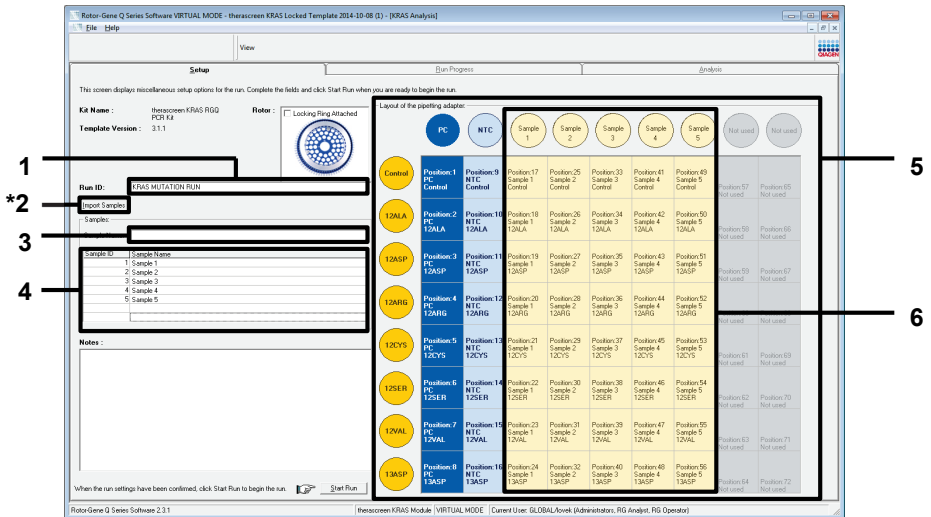
13. Start the Rotor-Gene Q software and open the template at the same time by double-clicking on the **therascreen KRAS Locked Template** icon on the desktop of the laptop connected to the Rotor-Gene Q instrument.



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 instrument.



15. Enter the run ID in the **Run ID** dialog field (1) 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.

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).

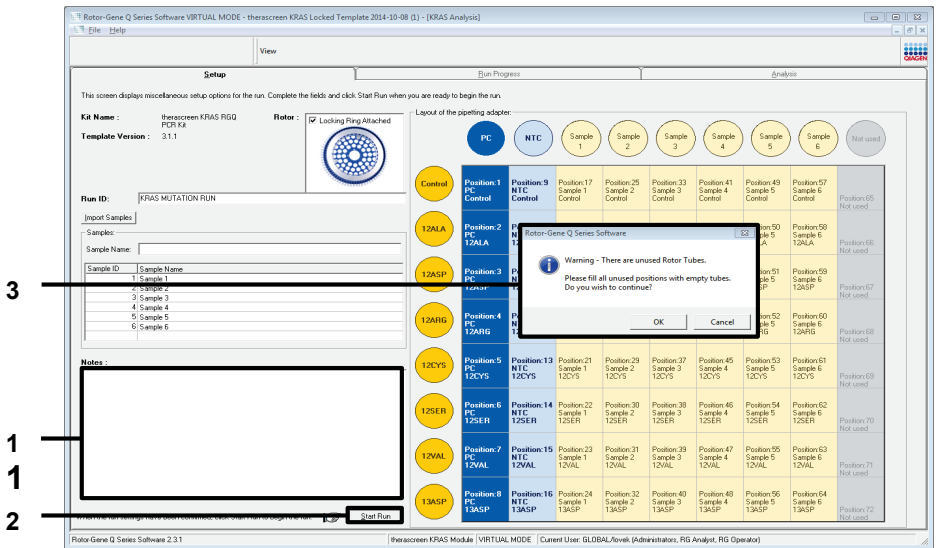
Note: A maximum of 7 samples can be added. The sample IDs (in the sample circles) will automatically be assigned from 1 to 7.

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

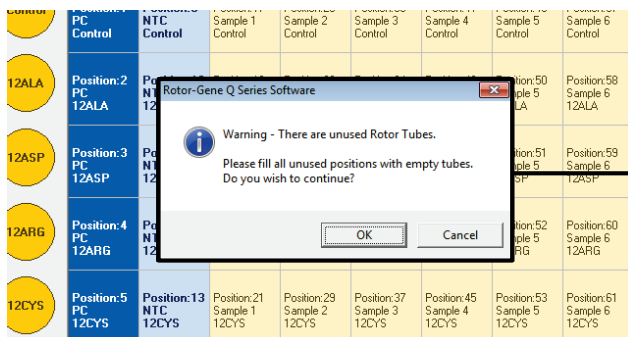
17. Repeat step 16 to enter the names of all additional samples.

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).



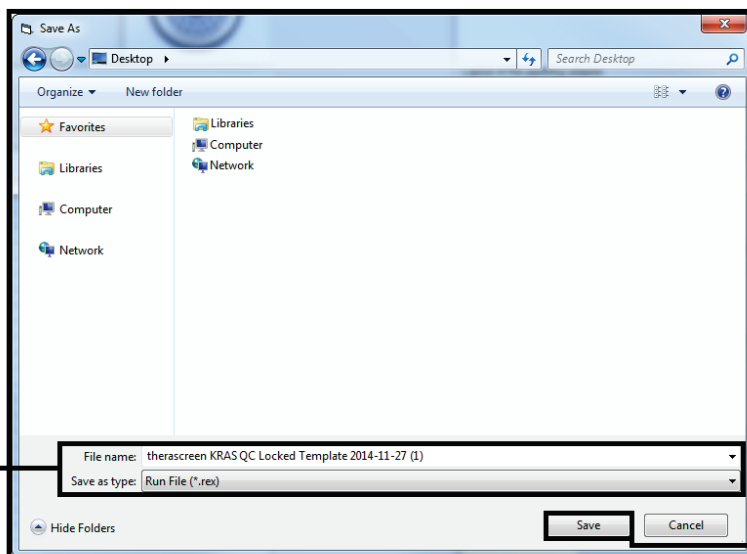
Note: If any rotor position is unused, a warning will appear (3 above and 1 in example below) to remind the user that all unused positions on the rotor must be filled with a capped, empty tube.



1

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.



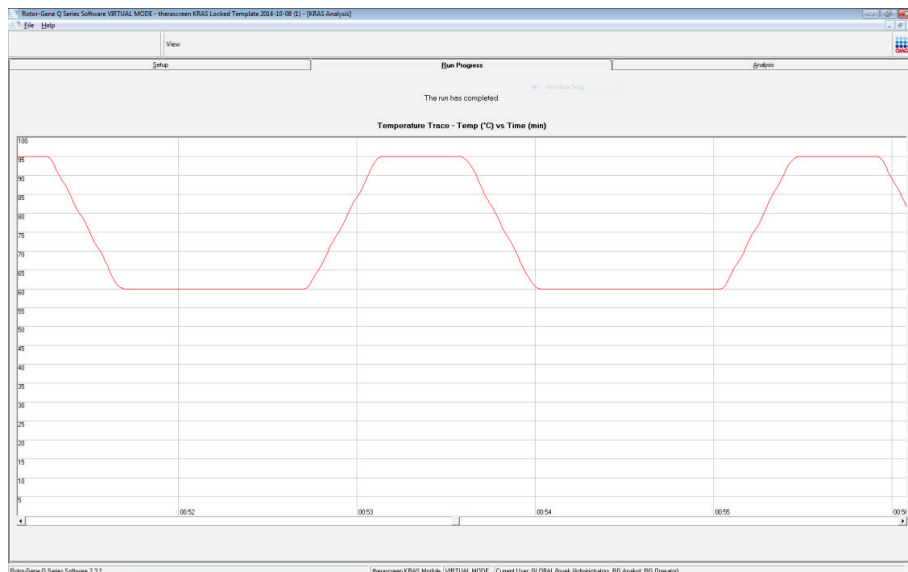
1

2

3

21. The PCR run starts.

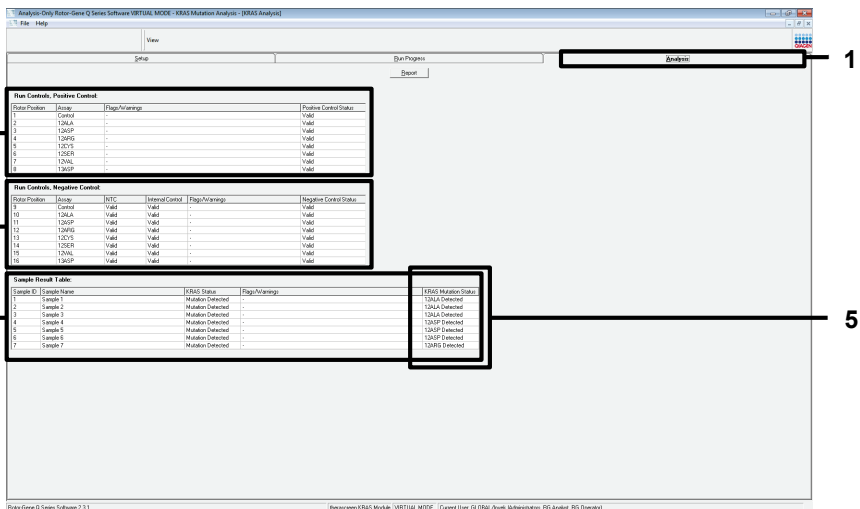
Note: When the run starts, the **Run Progress** tab will open automatically to show the temperature trace and remaining run time.



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 48.



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.

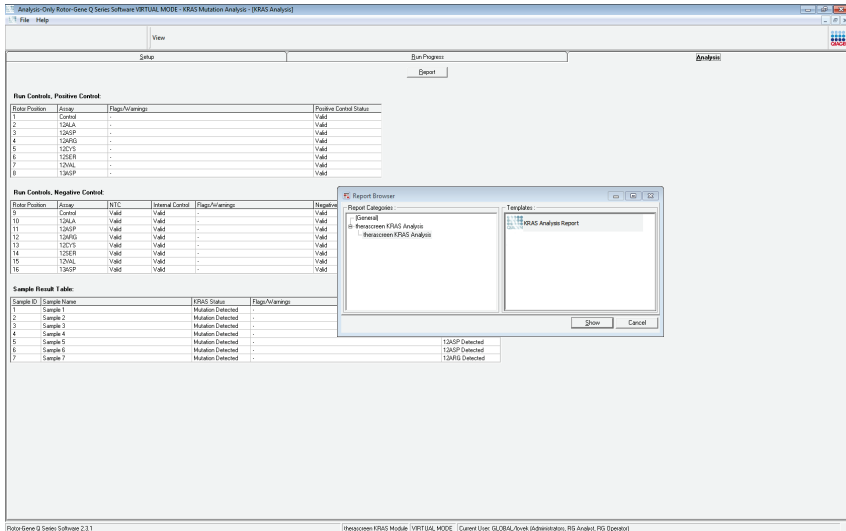
- The **Run Controls, Negative Control** panel (3)

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(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.
SAMPLE_INT_CTRL_FAIL	C _T too high (or no C _T) for internal control (HEX), FAM channel mutation-negative	<p>If sample given valid status — No action.</p> <p>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.</p>

Flag	Meaning	Action
SAMPLE_INT_CTRL_ EARLY_CT	Mutation tube invalid — C _T HEX too low for sample (internal control)	<p>If sample given valid status — No action.</p> <p>If sample given invalid status, 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.</p>

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.	<p>If sample given valid status — No action.</p> <p>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.</p>
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 contact information, see back cover or 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 "Reagent Storage and Handling," page 19 | Check the storage conditions and the expiration date (see the label) of the reagents and use a new kit, if necessary. |
| b) The <i>therascreen</i> KRAS RGQ PCR Kit has expired | Check the storage conditions and the expiration date (see the kit label) of the reagents and, if necessary, use a new <i>therascreen</i> 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.

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

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 (19). 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 3.

Table 3. 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 (20). 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 4.

Table 4. 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 5 (next page) shows details of the agreement between the *therascreen* KRAS RGQ PCR Kit and bidirectional sequencing.

Table 5. *therascreen* KRAS RGQ PCR Kit vs. Sanger bidirectional sequencing

KRAS Kit call	Mutation call by bidirectional sequencing											Total
	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	0	14	12	12	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 6 and Table 7).

Table 6. 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 7 (next page) shows details of the agreement between the *therascreen* KRAS RGQ PCR Kit and bidirectional sequencing in the second study.

Table 7. *therascreen* KRAS RGQ PCR Kit vs. Sanger bidirectional sequencing (second study)

KRAS Kit call	Mutation call by bidirectional sequencing										Total
	Negative	12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP			
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	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 8. Highlighted boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Table 8. Percentage of correct calls as a function of the dilution for each mutant 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 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 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 CT value 31) 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 low dilution series

Mutation	Mutation dilution high (%)						
	0.27	0.54	1.08	2.15	4.30	8.60	12.90
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 11.

Table 11. 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 12. 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 12. LOD values for each mutation assay using FFPE cell lines

Assay	LOD C₉₅ (percentage of mutant DNA in wild-type DNA)
12ALA	0.8
12ASP	2.6
12ARG	6.4
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 13. 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 13. 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 13 (continued).

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 $[2(-1/\text{slope})] - 1$. The amplification efficiency of the control compared to the mutant reaction indicates that the ΔC_T , and thus mutation call, is consistent across the working range of the assay. 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 14.

Table 14. Summary of the data on amplification efficiency (function of DNA input)

Sample	Control C _T	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	Difference in amplification efficiencies
12ALA	Control C _T	21.060	0.060	-1.008	0.007	-1.023	-0.993	0.989	0.03
	12ALA C _T	22.476	0.103	-0.987	0.013	-1.013	-0.961	1.019	
12ARG	Control C _T	20.825	0.083	-1.035	0.01	-1.056	-1.014	0.954	0.056
	12ARG C _T	23.237	0.083	-0.993	0.011	-1.016	-0.97	1.01	
12ASP	Control C _T	20.385	0.13	-1.013	0.16	-1.046	-0.98	0.982	-0.003
	12ASP C _T	21.347	0.065	-1.015	0.008	-1.032	-0.999	0.979	
12CYS	Control C _T	23.437	0.063	-0.981	0.01	-1.003	-0.96	1.026	0.032
	12CYS C _T	24.289	0.039	-0.961	0.006	-0.974	-0.947	1.058	
12SER	Control C _T	22.568	0.050	-1.003	0.008	-1.02	-0.986	0.996	0.105
	12SER C _T	25.212	0.087	-0.934	0.014	-0.963	-0.904	1.101	
12VAL	Control C _T	21.208	0.047	-0.995	0.006	-1.007	-0.983	1.007	0.033
	12VAL C _T	21.532	0.043	-0.972	0.005	-0.983	-0.961	1.04	
13ASP	Control C _T	23.207	0.056	-1.001	0.009	-1.02	-0.982	0.999	0.145
	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 $\pm 10\%$. A summary of the data is shown in Table 15.

Table 15. 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 16.

Table 16. Amount of interfering substances tested in each assay

Interfering substance	Actual high amount [$\mu\text{l}/200 \mu\text{l}$ eluate]	Actual low amount [$\mu\text{l}/200 \mu\text{l}$ 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 60), 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 17.

Table 17. 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 18 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 18. 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 (20, 21). 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 19 and Table 20.

Table 19. Assay variance estimates

Assay	%CV for ΔC_T		%CV for mutant C_T		%CV for control C_T		WT
	3xLOD	C50	3xLOD	C50	3xLOD	C50	
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 20. Repeatability precision estimates

Assay	%CV for ΔC_T		%CV for mutant C_T		%CV for control C_T		WT
	3xLOD	C50	3xLOD	C50	3xLOD	C50	
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 21).

Table 21. Correct calls overall

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

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 22 and Table 23). The results demonstrate the agreement of the DNA extraction procedure and sample processing in conjunction with the *therascreen* KRAS RGQ PCR Kit.

Table 22. 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 23. 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.

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 cetuximab (Erbixux)

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 cetuximab (Erbixux). The safety and effectiveness of cetuximab (Erbixux) 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 Erbixux plus best supportive care (BSC) or BSC alone. Erbixux was administered as a 400 mg/m² initial dose, followed by 250 mg/m² 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 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 cetuximab+BSC group and 5.0 (4.3, 5.7) months in the BSC group. The OS hazard ratio of 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 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 24 and Figure 1.

Table 24. Overall survival in previously treated EGFR-expressing metastatic colorectal cancer (all randomized and KRAS status)

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

* Based on the stratified log-rank test.

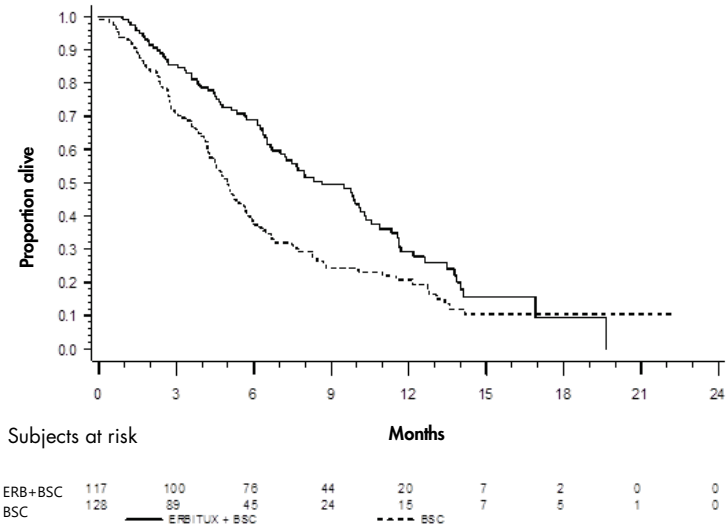


Figure 1. Kaplan-Meier curve for overall survival in patients with KRAS mutation-negative (wild-type) metastatic colorectal cancer.

Overall survival rates based on Kaplan-Meier estimates at months 6 and 12 were higher for the 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 panitumumab (Vectibix)

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 panitumumab (Vectibix). The objective of the study was to assess whether KRAS status as determined by the *therascreen* KRAS RGQ PCR Kit can be used to select patients with mCRC who will benefit from panitumumab (Vectibix) 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

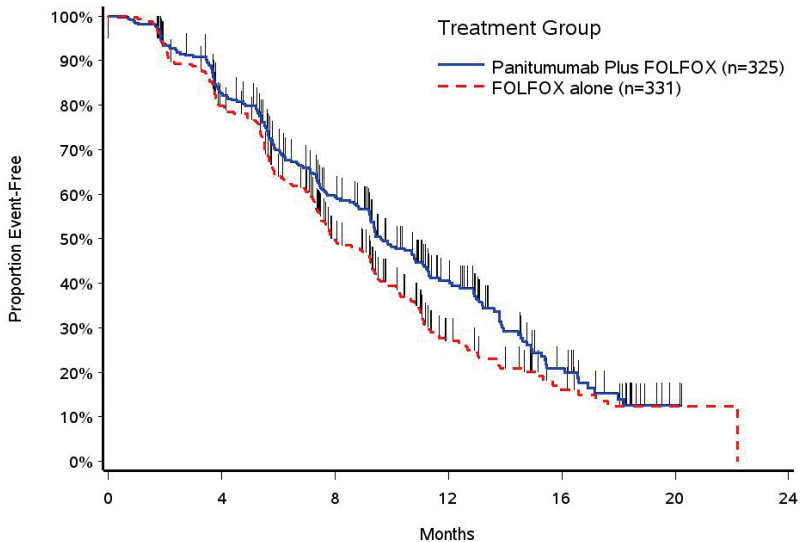
(5-FU) and leucovorin (FOLFOX) vs. FOLFOX alone in patients with previously untreated, recurrent mCRC.

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 oncogene 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 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 25 and Figure 2.

Table 25. Efficacy results in patients with wild-type KRAS mCRC

	Wild-type KRAS population	PFS			
		Median (months) (95% CI)	Hazard ratio (95% CI)	p-value	ORR (95% CI)
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%)



Subjects at risk:

Panitumumab Plus FOLFOX 325	254	156	73	22	1	0
FOLFOX alone 331	242	127	41	16	2	0

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 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 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 plus FOLFOX compared with FOLFOX alone. Median OS among 325 patients with wild-type KRAS mCRC who received Vectibix 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.

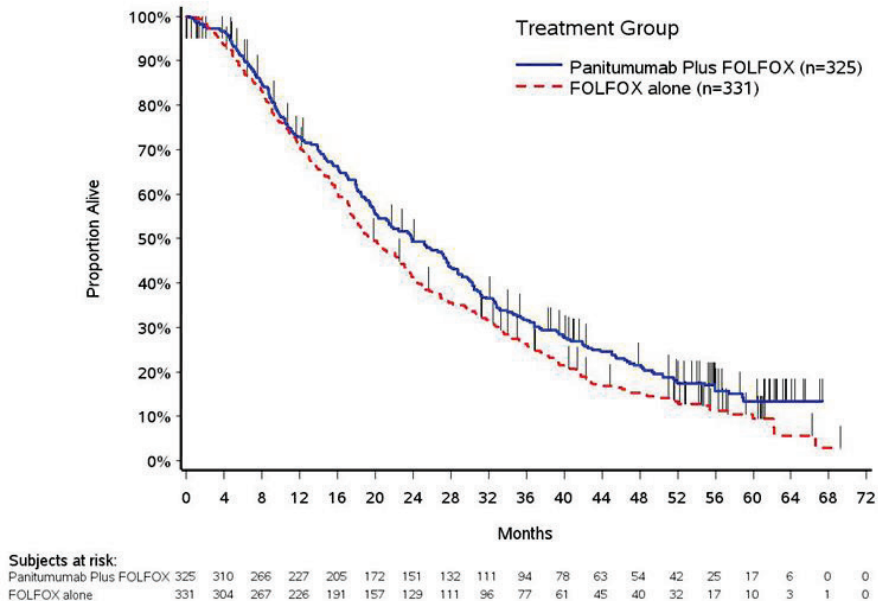


Figure 3. Kaplan-Meier Plot of overall survival (OS) in patients with Wild-type KRAS mCRC.

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

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Symbols

The following symbols may appear on the packaging and labeling:

Symbol

Symbol definition

IVD

In vitro diagnostic medical device

REF

Catalog number



Manufacturer

MAT

Material number

R_n

R is for revision of the Instructions for Use (Handbook) and n is the revision number

LOT

Lot number

GTIN

Global Trade Item Number



Use by








<24>

Contains reagents sufficient for 24 reactions

COMP

Components (i.e. a list of what is included)

Symbol	Symbol definition
	Contains (contents)
	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).

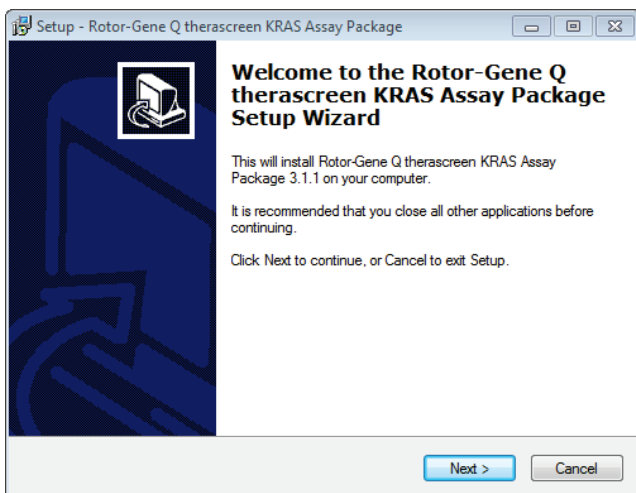
Note: The Rotor-Gene Q *therascreen* KRAS Assay Package software will only work with Rotor-Gene Q software version 2.3. Make sure the correct version of Rotor-Gene Q software is installed before proceeding with the *therascreen* KRAS Assay Package installation.

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.

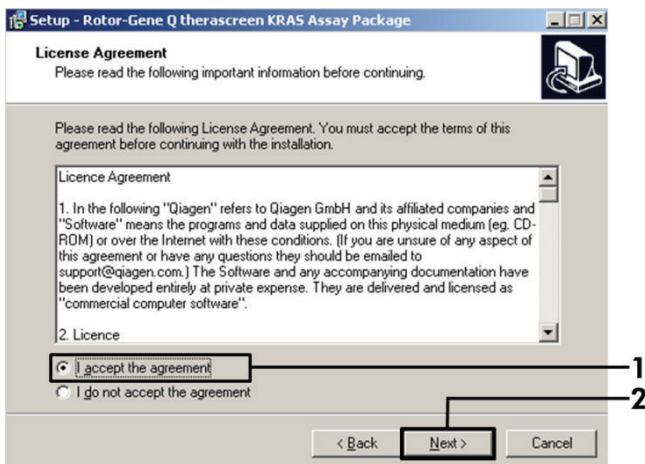
1. Order the Rotor-Gene Q *therascreen* KRAS RGQ Assay Package CD version 3.1.1 (QIAGEN, cat. no. 9023818) available separately from QIAGEN.
2. Insert the CD into the CD drive of the laptop connected to the Rotor-Gene Q MDx instrument.
3. Start the installation by double-clicking the file ***therascreen_KRAS_Assay_Package_3.1.1.exe***.

The setup wizard will appear.



4. Click **Next** to continue.

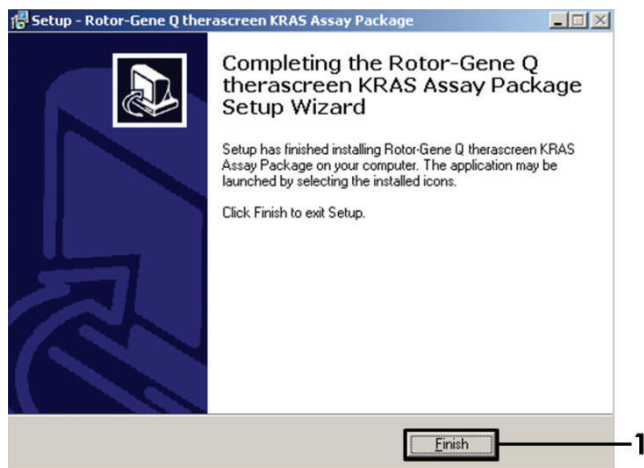
The **License Agreement** dialog box opens.



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 **thetascreen KRAS QC Locked Template** and **thetascreen KRAS Locked Template** will be generated automatically and appear on the desktop.

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> KRAS RGQ PCR Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, Water, Taq DNA Polymerase	870021
Rotor-Gene Q <i>therascreen</i> KRAS Assay Package 3.1.1 CD	Software protocol package for use with the <i>therascreen</i> 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
Strip Tubes and Caps, 0.1 ml (2500)	10 x 250 strips of 4 tubes and caps for 10,000 reactions	981106

Product	Contents	Cat. no.
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

Trademarks: QIAGEN[®], Sample to Insight[®], QIAamp[®], QIAcube[®], MinElute[®], Rotor-Gene[®], Scorpions[®], *therascreen*[®] (QIAGEN Group); Erbitux[®] (ImClone LLC., a wholly-owned subsidiary of Eli Lilly and Company); Vectibix[®] (Immunex Corp.), FAM[™], HEX[™] (Life Technologies, Inc.).

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