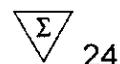


Draft 4th July 2012

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



Version 1

For in vitro diagnostic use

For use with Rotor-Gene[®] Q MDx instrument

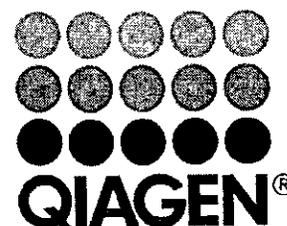
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Intended Use

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 seven 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) based on a KRAS no mutation detected test result.

Summary and Explanation of the Test

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, 5–8). Such mutations in the KRAS oncogene are present in around 40% of cases (9).

Using Scorpions[®] (10) and ARMS[®] (Allele Refractory Mutation System) technologies (11, 12), 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 (see 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. (13).

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 eight separate PCR amplification reactions: seven 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 bi-functional molecules containing a PCR primer covalently linked to a probe. The probe incorporates the fluorophore carboxyfluorescein (FAM™) and a quencher. The latter quenches the fluorescence of the fluorophore. When the probe binds to the ARMS amplicon during PCR, the fluorophore and quencher become separated, leading to a detectable increase in fluorescence.

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 eight reaction mixes contains an internal control designed to detect failure of the reaction (e.g., due to the presence of inhibitors). The internal control employs a non-KRAS related oligonucleotide target sequence, an unlabeled primer, and a Scorpions primer labeled with

hexachlorofluorescein (HEX™) in order to distinguish it from the FAM-labeled Scorpions in the control and mutation reactions.

Positive control

The *therascreen* KRAS RGQ PCR Kit also contains the KRAS Positive Control (tube PC). The Positive Control is comprised of 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" (NTC) reaction. The NTC 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 to be used with the Rotor-Gene Q MDx instrument. 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) and 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 CT 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 pre-determined analytical C_T and ΔC_T values, the Rotor-Gene Q MDx software qualitatively determines the mutation status of the DNA samples and reports which samples contain which mutation.*

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

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

Table continued on next page.

Table 2. Continued.

Reagent	Ingredients	
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 μ l
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 μ l
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 μ l
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 μ l
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 μ l
Taq DNA Polymerase	Taq Polymerase, 50% glycerol/water	1 tube x 70 μ l
No template control	Nuclease-Free Water	1 tube x 1.9 ml
For template dilution	Nuclease-Free Water	1 tube x 1.9 ml

Platform

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 version 1.0.12, available separately on CD (QIAGEN, cat. no. 9021168).

- Refer to the instrument user manual for information concerning the instrument.
- Refer to "Appendix A: Installation of the Rotor-Gene Q *therascreen* KRAS Assay Package", page 81, for instructions on installing the Rotor-Gene Q MDx *therascreen* KRAS Assay Package.

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

Warnings and Precautions

For in vitro diagnostic use.

For prescription use only.

Safety information

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

General Precautions

- 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 μ l.
- 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 *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 instrument 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, 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 one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

The *therascreen* KRAS RGQ PCR Kit should be stored immediately upon receipt at -15 to -25°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 -15 to 25°C for 4 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 immediately. The total time prior to run once the PCR reactions are set up should not exceed:

- 7 hours if stored at room temperature

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

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

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

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

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

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 Collection, Preparation for Analysis, and Storage

The *therascreen* KRAS RGQ PCR Kit is for use on DNA samples extracted from formalin-fixed paraffin-embedded (FFPE) tumor tissue collected from colorectal cancer (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 macro-dissection (see below).
- For sections that are ≤20% tumor content by area, macrodissect one or more sections. Discard the non-tumor tissue.
- For sections that are <4mm² in area, process two or more sections to increase the total tumor area to at least 4mm² (applies to samples both with and without macro-dissection). Discard the non-tumor tissue.
- Scrape excess paraffin away from the tissue using a fresh, sterile scalpel.

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

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

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

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 –15 to –25°C up to 8 weeks before use.

Materials Provided

Kit contents

Table 3

<i>therascreen</i> KRAS RGQ PCR Kit				(24)
Catalog no.				870021
Number of preps				24
Color	Identity	Tube identification		Volume
Red	Control Reaction Mix	1	CTRL	2 x 600 μ l
Purple	12ALA Reaction Mix	2	12ALA	600 μ l
Orange	12ASP Reaction Mix	3	12ASP	600 μ l
Pink	12ARG Reaction Mix	4	12ARG	600 μ l
Green	12CYS Reaction Mix	5	12CYS	600 μ l
Yellow	12SER Reaction Mix	6	12SER	600 μ l
Grey	12VAL Reaction Mix	7	12VAL	600 μ l
Blue	13ASP Reaction Mix	8	13ASP	600 μ l
Beige	KRAS Positive Control	9	PC	250 μ l
Mint	<i>Taq</i> DNA Polymerase		<i>Taq</i>	70 μ l
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 material safety data sheets (MSDSs), available from the product supplier.

- 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 2.1.0

Note: The Rotor-Gene Q MDx *therascreen* KRAS Assay Package software will only work with the software version 2.1.0 (Build 9) or higher.

- Rotor-Gene Q MDx *therascreen* KRAS Assay Package CD version 1.0.12 (QIAGEN, cat. no. 9021168)
- Loading Block 72 x 0.1 ml Tubes; aluminum block for manual reaction setup (QIAGEN; cat. no. 9018901)
- Dedicated pipets* (adjustable) for sample preparation
- Dedicated pipets* (adjustable) for PCR master mix preparation
- Dedicated pipets* (adjustable) for dispensing of template DNA
- Thermomixer,* heated orbital incubator, heating block, or water bath capable of incubation at 56°C 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 17)

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

Procedure

DNA extraction

Use the QIAamp DSP DNA FFPE Tissue Kit (QIAGEN, cat. no. 60404) with the modifications to 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*.
- 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 into 200 µl of elution 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 –15 to –25°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

- 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.

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.

- 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

- Ensure that the Rotor-Gene Q *therascreen* KRAS Assay Package software is installed before first use of the Rotor-Gene Q MDx instrument (see "Appendix A: Installation of the Rotor-Gene Q *therascreen* KRAS Assay Package", page 81).
- 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.

Procedure

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 Table 4. 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.

Table 4. Thawing times, PCR setup times, and storage temperatures

Thaw time		Storage temperature after PCR setup	Maximum PCR setup and storage time
Minimum	Maximum		
1 hour	4.5 hours	Room temperature (15–25°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 12). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

2. Prepare sufficient 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 Table 5. Include reagents for 2 extra samples to allow sufficient overage for the PCR setup.

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

Table 5. Preparation of Control assay master mix

Component	Volume
Control Reaction Mix (tube CTRL)	19.76 μ l x (n+2)*
Taq DNA polymerase (tube Taq)	0.24 μ l x (n+2)*
Total volume	20.0 μ l/reaction

* n=number of reactions (samples plus controls). Prepare enough master mix for 2 extra samples (n+2) to allow for sufficient overage for the PCR setup. The value n should not exceed 24 (plus controls) as 24 is the maximum number of samples which can fit on a run.

Note: When preparing the master mix, the required volume of the Control Reaction Mix 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 four tubes) in the loading block according to the layout in Table 6. Do not cap the tubes.

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

Table 6. Run layout for DNA sample assessment in the loading block*

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	-	-	-	-	-	-

* Each tube should contain a total reaction volume of 25 μ l (20 μ l of master mix prepared in Table 5, plus 5 μ l of NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position.

4. Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the master mix by fully aspirating up and down 10 times. Immediately add 20 μ l master mix to each PCR strip tube.

Note: Refer to Table 6 for the tube layout while setting up the reaction mixes. 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.

5. Immediately add 5 μ l of nuclease-free water for No Template Control (tube NTC) to the NTC tube (tube position 2) and cap the tube.
6. Add 5 μ l of each DNA sample to the sample tubes (tube positions 3-26) and cap the tubes.
7. Add 5 μ l of KRAS Positive Control (tube PC) to the PC tube (tube position 1) and cap the tube.
8. 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.
9. Invert capped tubes 4 times to mix the sample and reaction mix.
10. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 6) 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.

11. 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.
12. Open the Rotor-Gene Q MDx software according to step 13.
13. Start the Rotor-Gene Q MDx 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 (Figure 1).



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

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

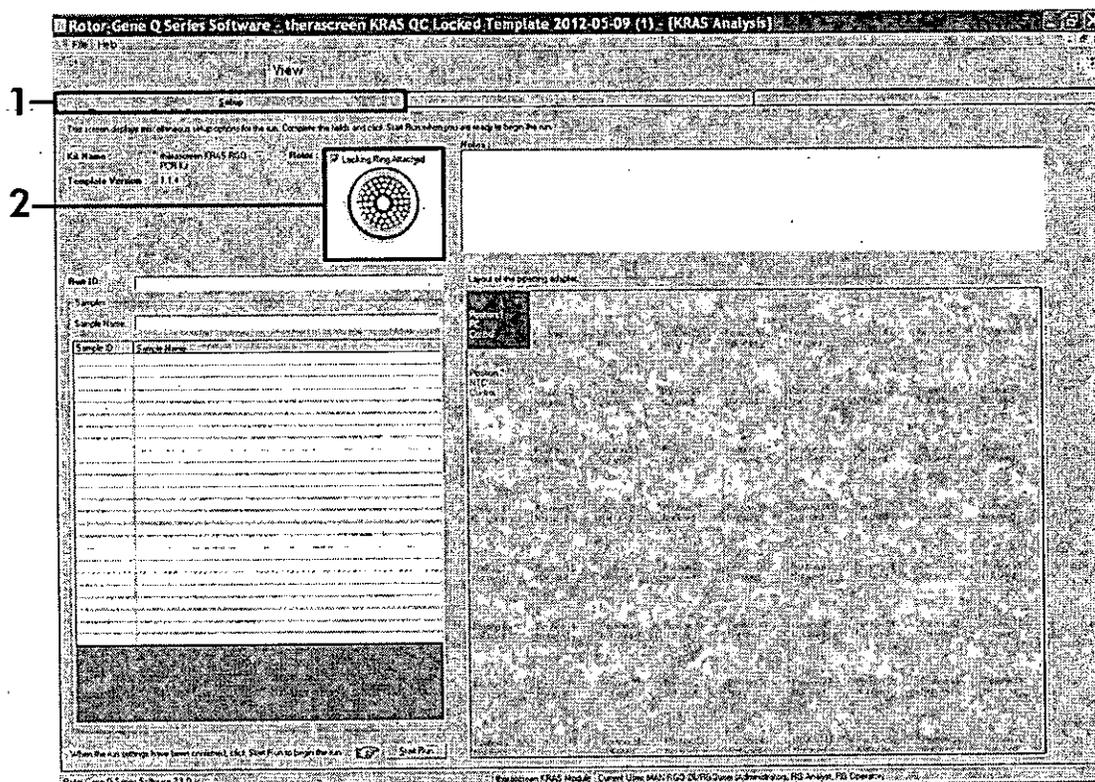


Figure 2. The “Setup” tab and “Locking Ring Attached” box. 1 = “Setup” tab, 2 = “Locking Ring Attached” box.

15. Enter the run ID in the “Run ID” dialog field according to your local naming convention. Enter the sample name in the “Sample Name” dialog field according to your local naming convention and press the return key. This will add the sample name to the sample list below and assign the sample a “Sample ID” (1, 2, 3, etc.). In addition, the “Layout of the pipetting adapter” panel on the right side will update to include the sample name (Figure 3).

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 (Figure 3).

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

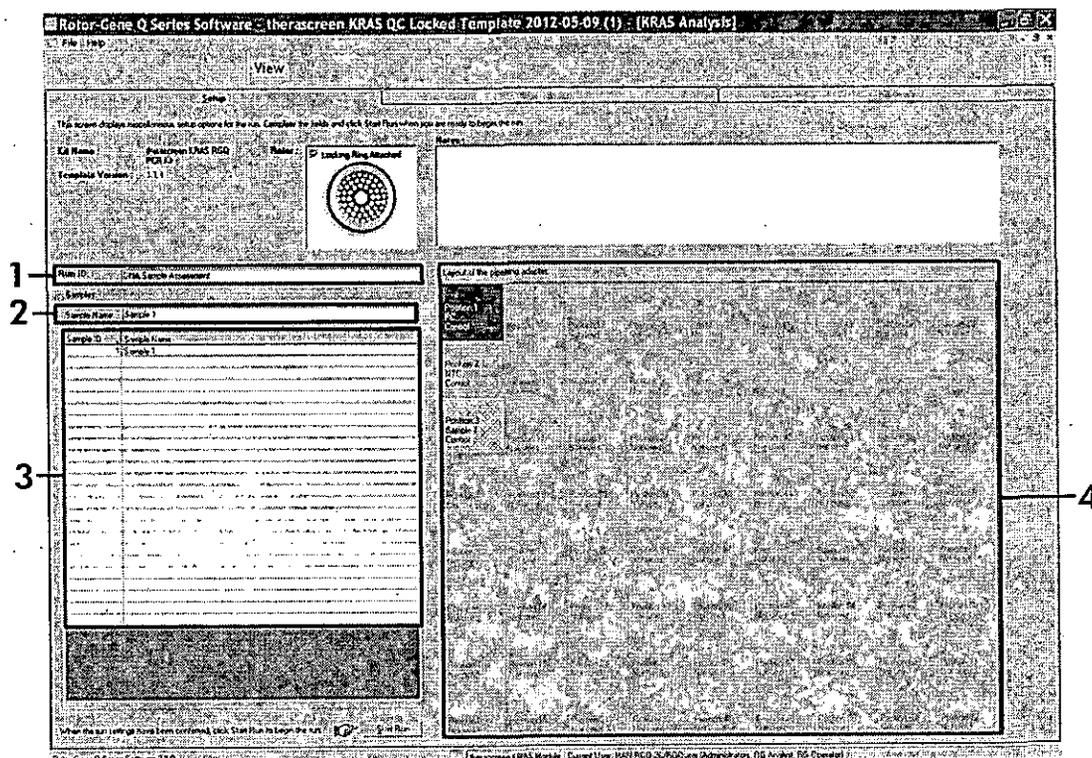


Figure 3. Entering the "Run ID" and "Sample Name". 1 = "Run ID" dialog field, 2 = "Sample Name" dialog field, 3 = Sample List, 4 = "Layout of the pipetting adapter" panel.

16. Repeat step 15 to enter the names of all additional samples (Figure 4).

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

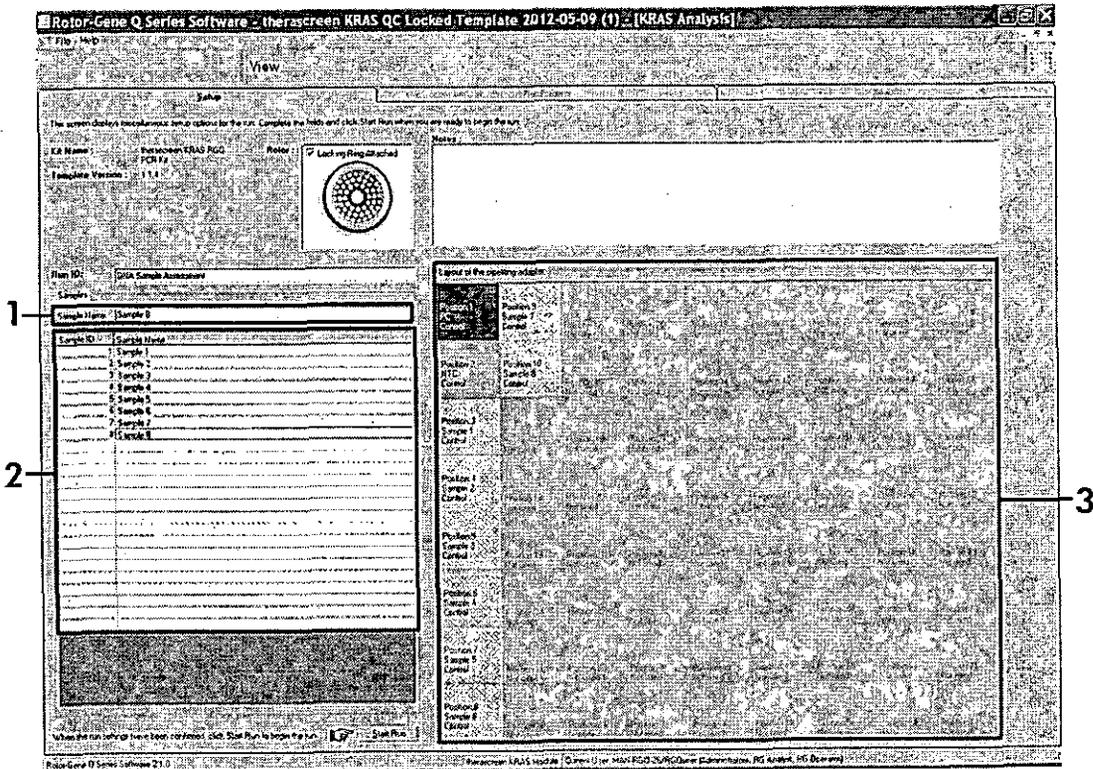


Figure 4. Entering additional sample names in the "Sample Name" dialog field. 1 = "Sample Name" dialog field, 2 = Sample List, 3 = "Layout of the pipetting adapter" panel with additional sample names.

17. When all sample names have been entered, verify they are correct.

Add any additional information in the "Notes" dialog field if necessary and then click the "Start Run" button (Figure 5).

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

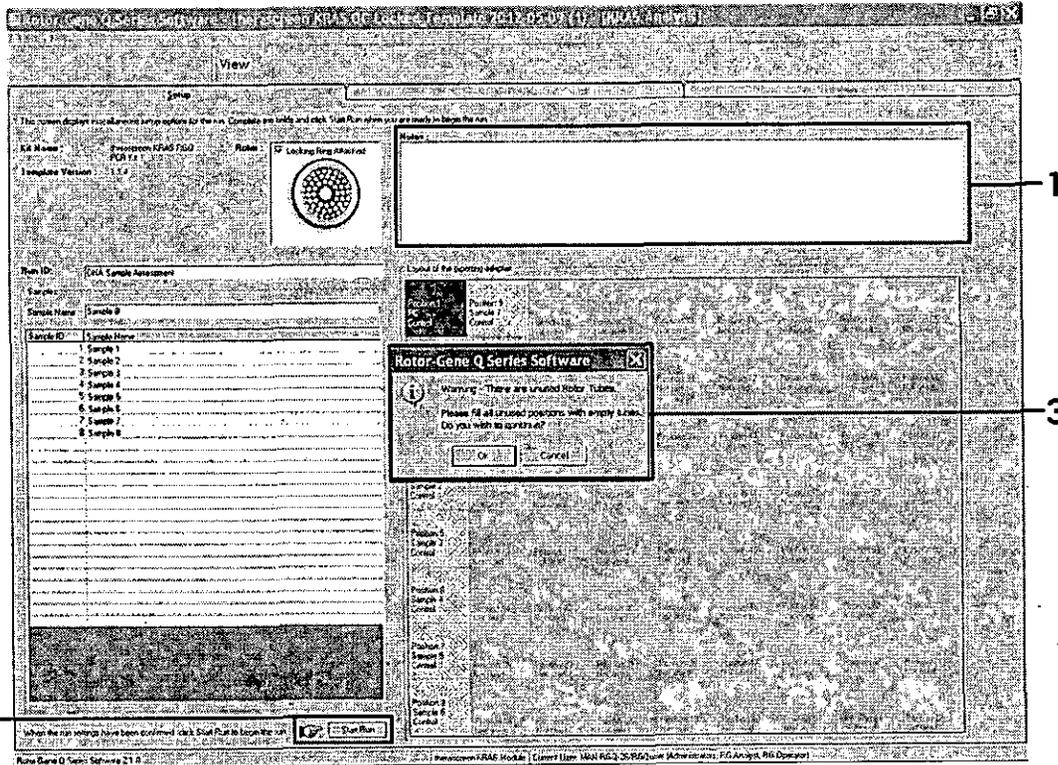


Figure 5. "Notes" dialog field, the "Start Run" button and "Warning" of unused rotor positions. 1 = "Notes" dialog field, 2 = "Start Run" button, 3 = "Warning" of unused rotor positions.

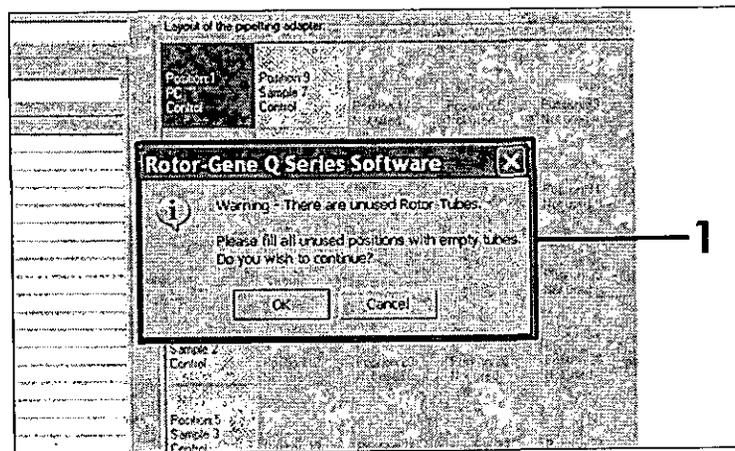


Figure 6. "Warning" of unused rotor positions. 1 = "Warning" of unused rotor positions.

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

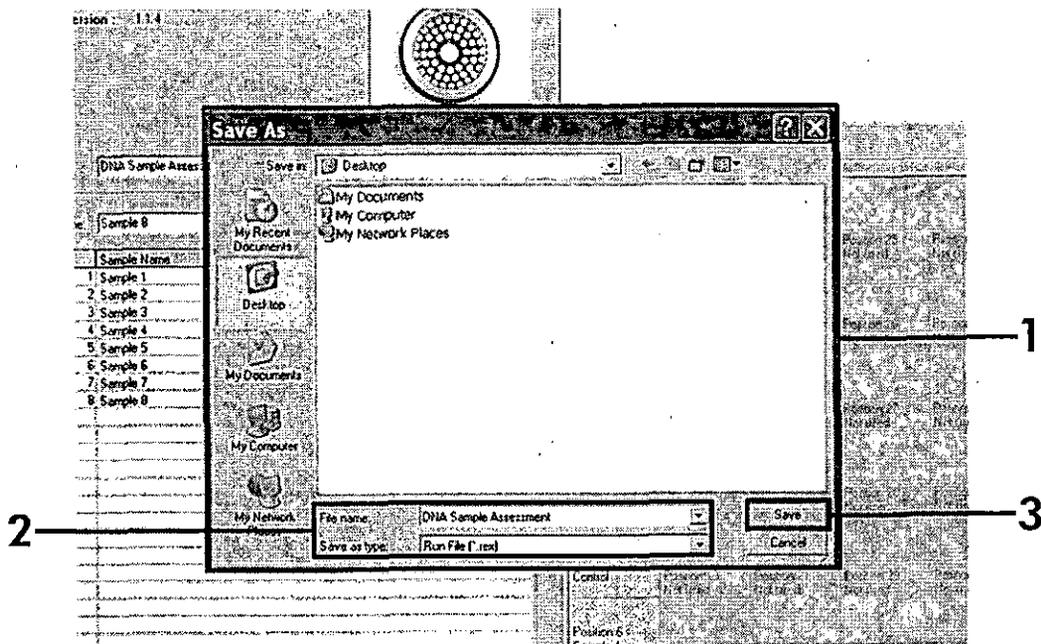


Figure 7. Saving the run file. 1 = "Save As" window, 2 = File name and save as type *.rex file, 3 = "Save" button.

19. The PCR run starts.

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

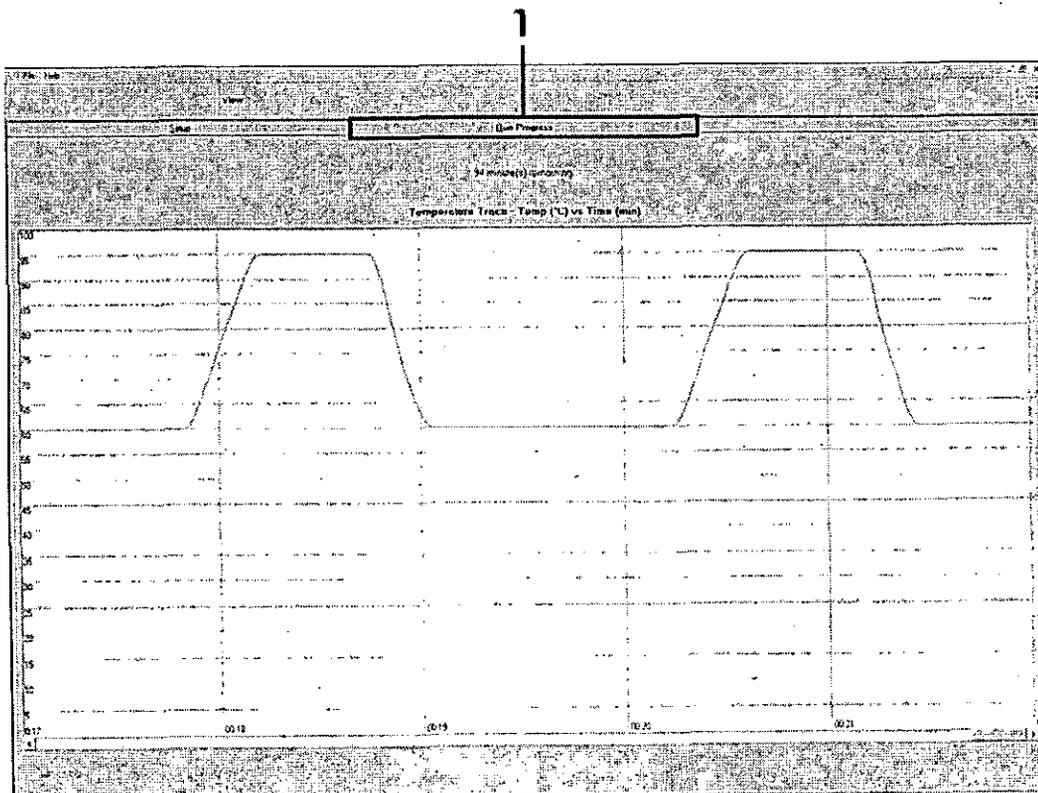


Figure 8. The "Run Progress" tab. 1 = "Run Progress" tab.

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

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

Sample ID	Sample Name	Control Array ID	Fluor/Volume	Status
1	PC Control	3510		Valid
2	HTC Control	3510		Valid
3	037710208	29.71		Valid
4	037710218	27.38		Valid
5	037710228	31.07		Valid
6	037710238	35.55		Valid
7	037710248	29.55		Valid
8	037710258	29.45		Valid
9	037710268	29.95		Valid
10	037710278	29.02		Valid
11	037710288	31.42		Valid
12	037710298	33.81		Valid
13	037710308	33.65		Valid
14	037710318	31.44		Valid
15	037710328	31.02		Valid
16	037710338	28.09		Valid
17	037710348	28.91		Valid
18	037710358	30.31		Valid
19	037710368	30.32		Valid
20	037710378	27.17		Valid
21	037710388	23.87		Valid
22	037710398	25.32		Valid
23	037710408	28.22		Valid
24	037710418	28.57		Valid
25	037710428	29.90		Valid
26	037710438	30.41		Valid

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

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

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

- Run controls (PC and NTC, tube positions 1 and 2 respectively). If the results are within acceptable ranges, each will display "Valid", otherwise an "Invalid" result will appear.
- Sample control reaction $C_T > 32.00$, will display "Invalid". Quantity of DNA is not sufficient for mutation analysis. Retest the sample. If the quantity of DNA is still insufficient, extract more tumor tissue if available (see "Troubleshooting Guide", page 89).
- Sample control reaction $C_T < 21.92$, will display "Invalid". 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 of 21.92–32.00, ($21.92 \leq \text{Control } C_T \leq 32.00$) will display "Valid". DNA concentration is suitable for mutation analysis.

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

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

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

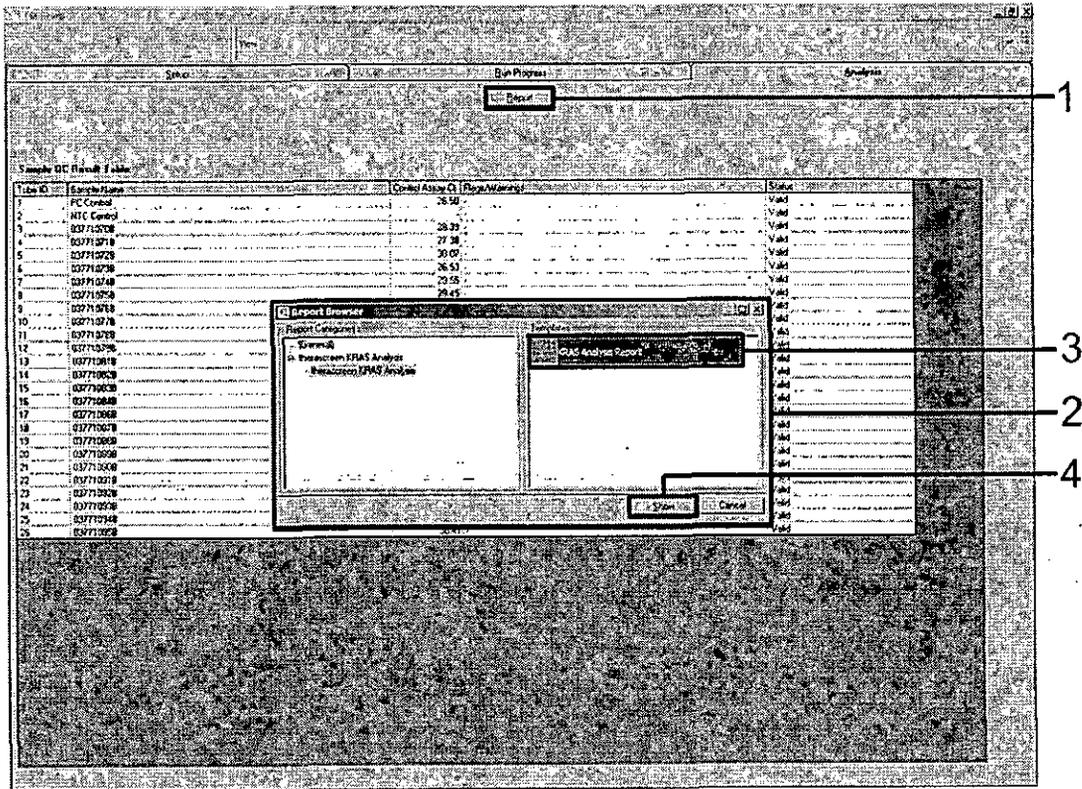


Figure 10. Selecting the "KRAS Analysis Report". 1 = "Report" button, 2 = "Report Browser" window, 3 = "KRAS Analysis Report" selection, 4 = "Show" button.

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.
- 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.

Protocol

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 Table 7. 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.

Table 7. Thawing times, PCR setup times, and storage temperatures

Thaw time		Storage temperature after PCR setup	Maximum PCR setup and storage time
Minimum	Maximum		
1 hour	4.5 hours	Room temperature (15–25°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 the *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 12). Centrifuge the tube briefly to collect the enzyme at the bottom of the tube.

- Label eight microcentrifuge tubes (not provided) according to each corresponding reaction mix shown in Table 8. 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 Table 8. Include reagents for 2 extra samples to allow sufficient overage for the PCR setup.

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

Table 8. Preparation of assay master mixes*

Assay	Reaction mix tube	Volume of reaction mix	Volume of <i>Taq</i> DNA polymerase (tube <i>Taq</i>)
Control	CTRL	19.76 μ l x (n+2)	0.24 μ l x (n+2)
12ALA	12ALA	19.76 μ l x (n+2)	0.24 μ l x (n+2)
12ASP	12ASP	19.76 μ l x (n+2)	0.24 μ l x (n+2)
12ARG	12ARG	19.76 μ l x (n+2)	0.24 μ l x (n+2)
12CYS	12CYS	19.76 μ l x (n+2)	0.24 μ l x (n+2)
12SER	12SER	19.76 μ l x (n+2)	0.24 μ l x (n+2)
12VAL	12VAL	19.76 μ l x (n+2)	0.24 μ l x (n+2)
13ASP	13ASP	19.76 μ l x (n+2)	0.24 μ l x (n+2)

* n=number of reactions (DNA samples plus controls). Prepare enough for 2 extra samples (n+2) to allow for sufficient coverage 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 master mix, the required volume of the control or mutation reaction mix is added to the relevant tube first and the *Taq* DNA polymerase is added last.

- Place the appropriate number of PCR 4-strip tubes (each strip has four tubes) in the loading block according to the layout in Table 6. Do not cap the tubes.

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

- Set a pipet to a volume lower than the total reaction mix volume and thoroughly mix the master mixes by fully aspirating up and down 10 times. Immediately add 20 μ l master mix to each appropriate PCR strip tube.

Note: Refer to Table 9 for the tube layout while setting up the reaction mixes. For detection of KRAS mutations, master mixes should be added to eight PC tubes, eight NTC tubes, and eight tubes for each DNA sample.

Table 9. Run layout for detection of KRAS Mutations in the loading block*

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
13ASP	8	16	24	32	40	48	56	64	72

* Each tube should contain a total reaction volume of 25 μ l (20 μ l of master mix prepared in Table 5, plus 5 μ l of NTC/sample/PC). Numbers denote positions in the loading block and indicate final rotor position.

5. 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.
6. Add 5 μ l of each DNA sample to the sample tubes (tube positions 17-72) and cap the tubes.
7. Add 5 μ l of KRAS Positive Control (tube PC) to the PC tubes (tube positions 1-8) and cap the tubes.
8. 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.
9. Invert the capped tubes 4 times to mix the sample and reaction mix.
10. Place all PCR 4-strip tubes into the appropriate positions of the 72-well rotor according to the run layout (Table 6) 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.

11. 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.
12. Open the Rotor-Gene Q MDx software according to step 13
13. Start the Rotor-Gene Q MDx 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 (Figure 11).



Figure 11. The "*therascreen* KRAS Locked Template" icon.

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

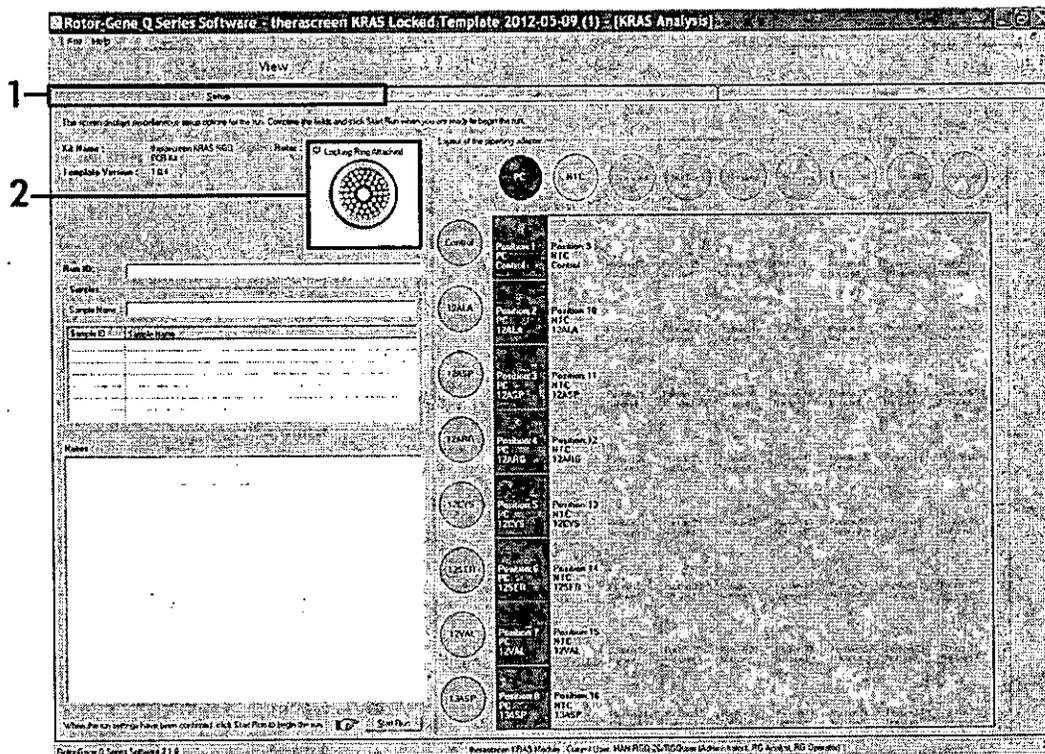


Figure 12. The "Setup" tab and "Locking Ring Attached" box. 1 = "Setup" tab, 2 = "Locking Ring Attached" box.

15. Enter the run ID in the "Run ID" dialog field according to your local naming convention. Enter the sample name in the "Sample Name" dialog field according to your local naming convention and press the return key. This will add the sample name to the sample list below and assign the sample a "Sample ID" (1, 2, 3, etc.). In addition, the "Layout of the pipetting adapter" panel on the right side will update to include the sample name (Figure 13).

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

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

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

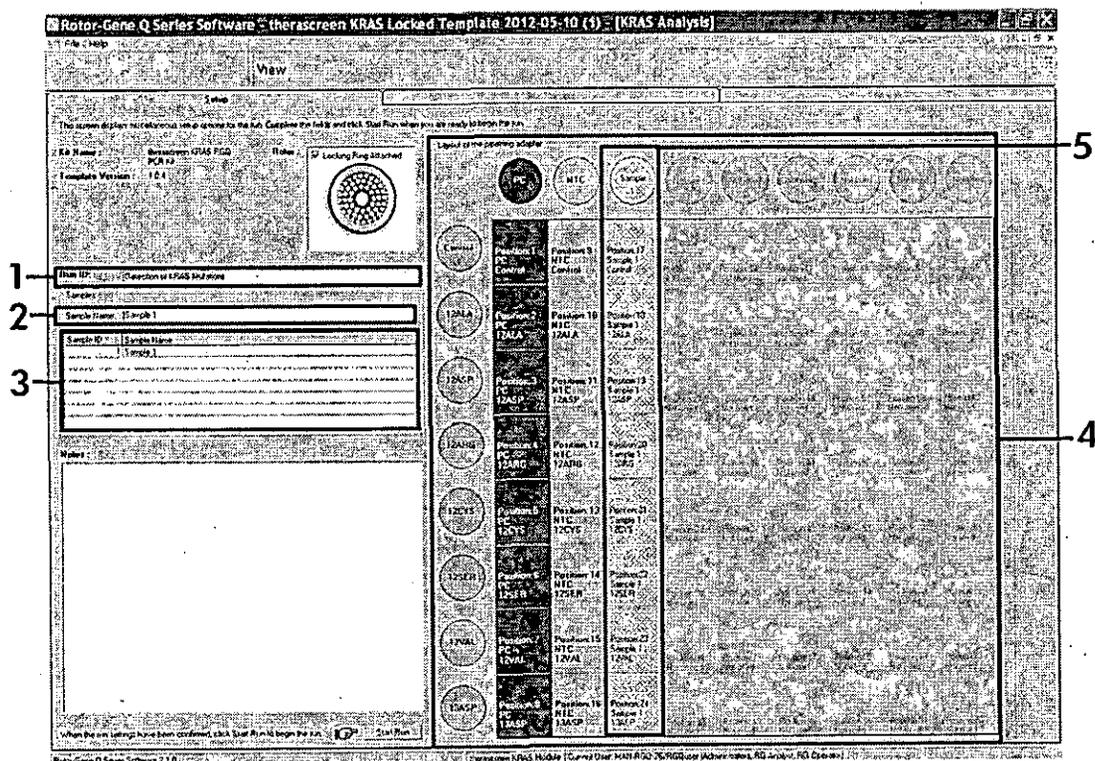


Figure 13. Entering the "Run ID" and "Sample Name". 1 = "Run ID" dialog field, 2 = "Sample Name" dialog field, 3 = Sample List, 4 = "Layout of the pipetting adapter" panel, 5 = Highlighted sample circle and column of eight assays underneath.

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

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

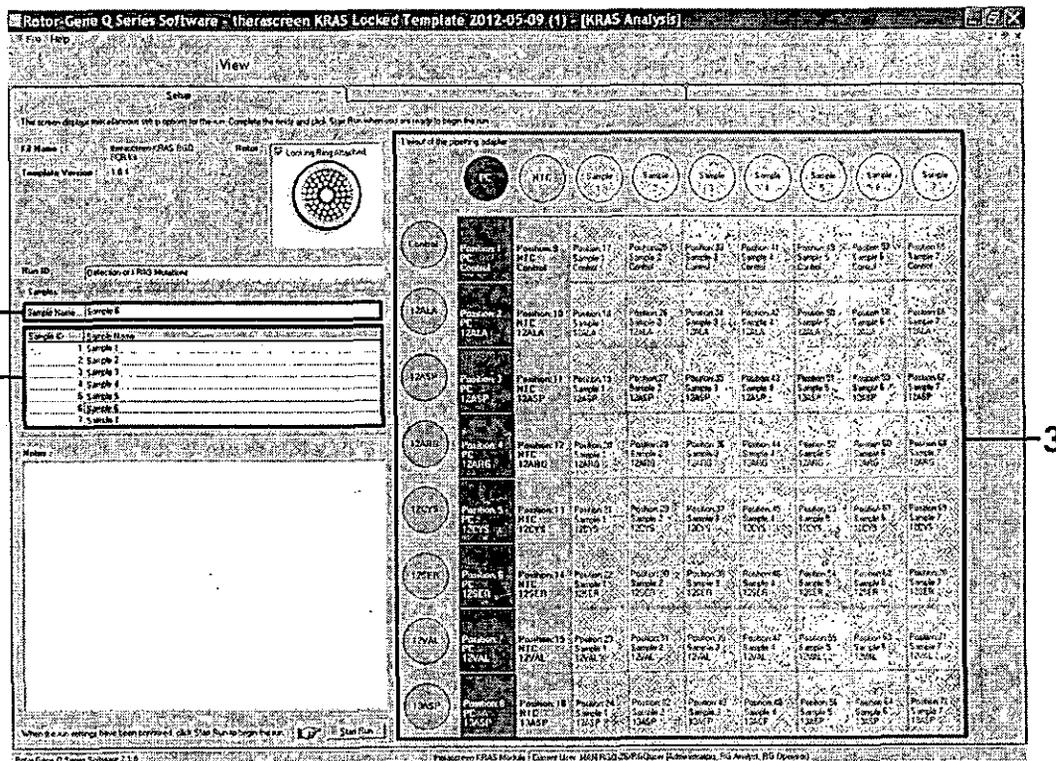


Figure 14. Entering additional sample names in the "Sample Name" dialog field. 1 = "Sample Name" dialog field, 2 = Sample List, 3 = "Layout of the pipetting adapter" panel with additional sample names.

17. When all sample names have been entered, verify they are correct.

Add any additional information in the "Notes" dialog field if necessary and then click the "Start Run" button (Figure 15).

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

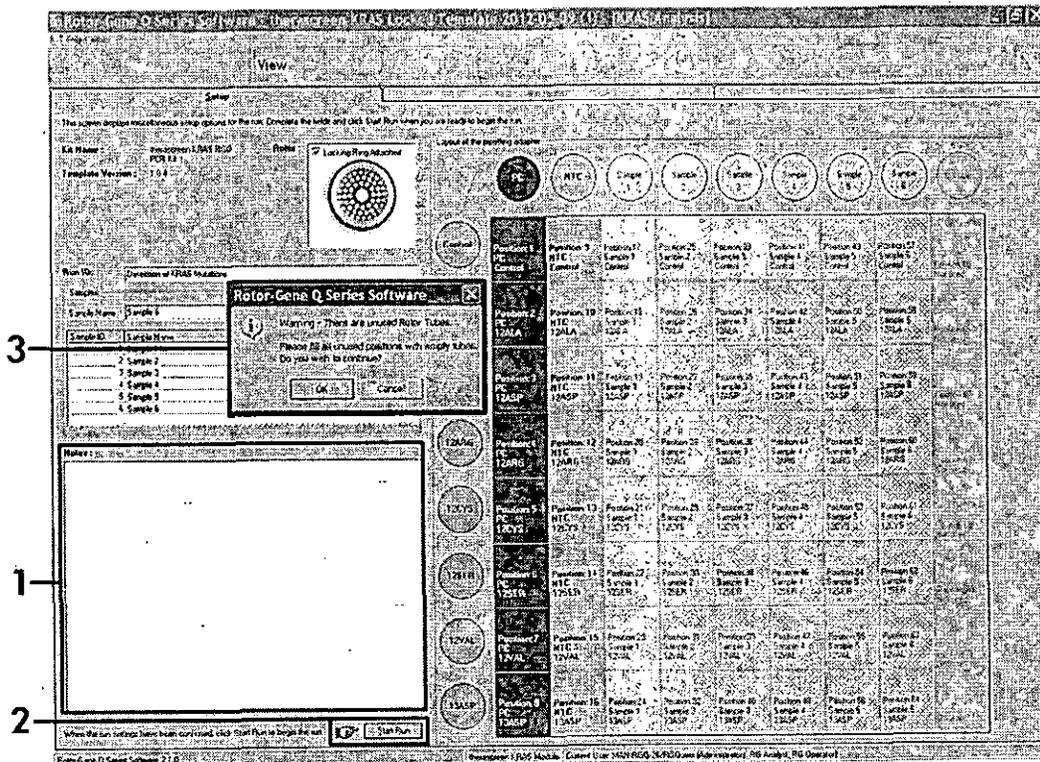


Figure 15. "Notes" dialog field, the "Start Run" button and "Warning" of unused rotor positions. 1 = "Notes" dialog field, 2 = "Start Run" button, 3 = "Warning" of unused rotor positions.

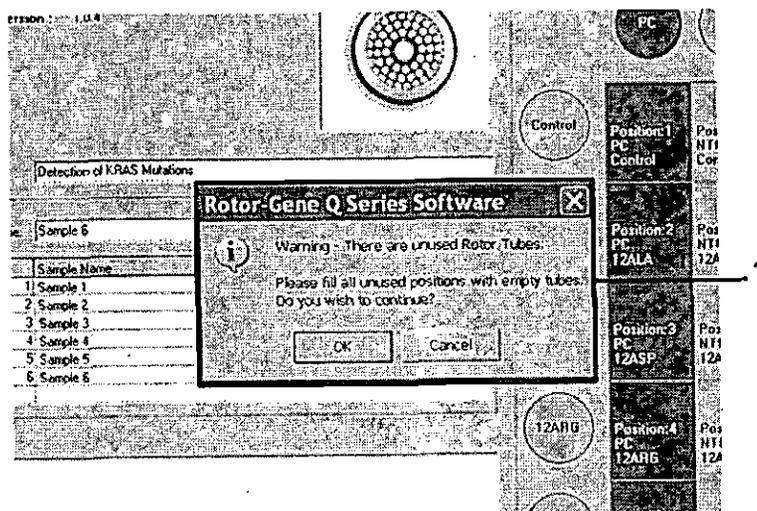


Figure 16. "Warning" of unused rotor positions. 1 = "Warning" of unused rotor positions.

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

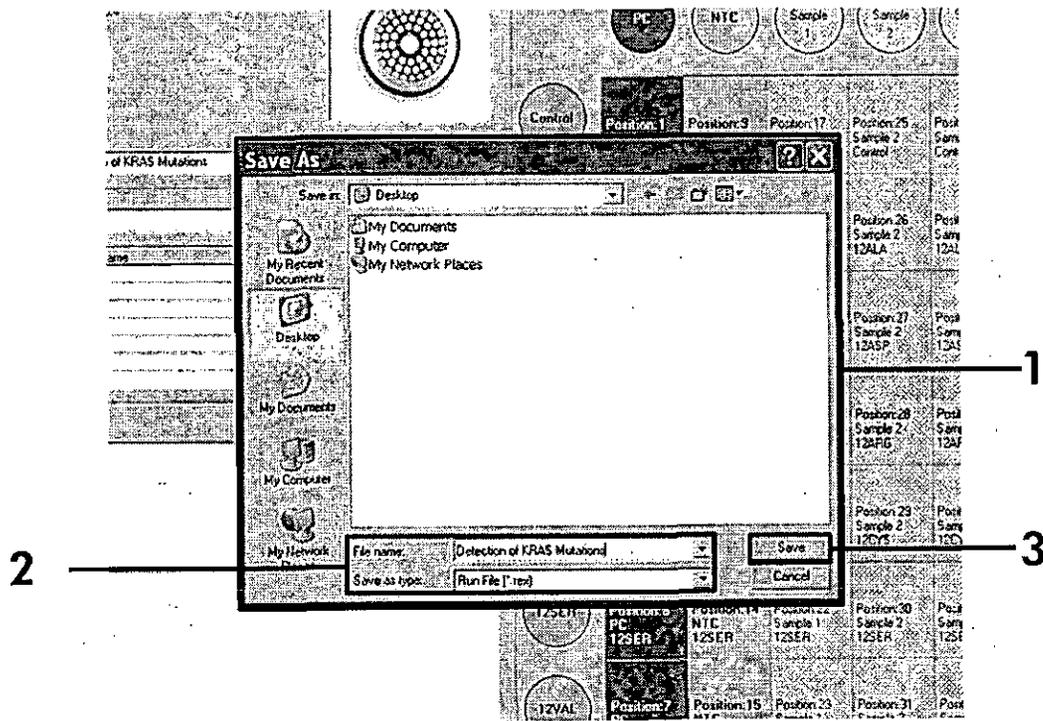


Figure 17. Saving the run file. 1 = "Save As" window, 2 = File name and save as type *.rex file, 3 = "Save" button.

19: The PCR run starts.

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

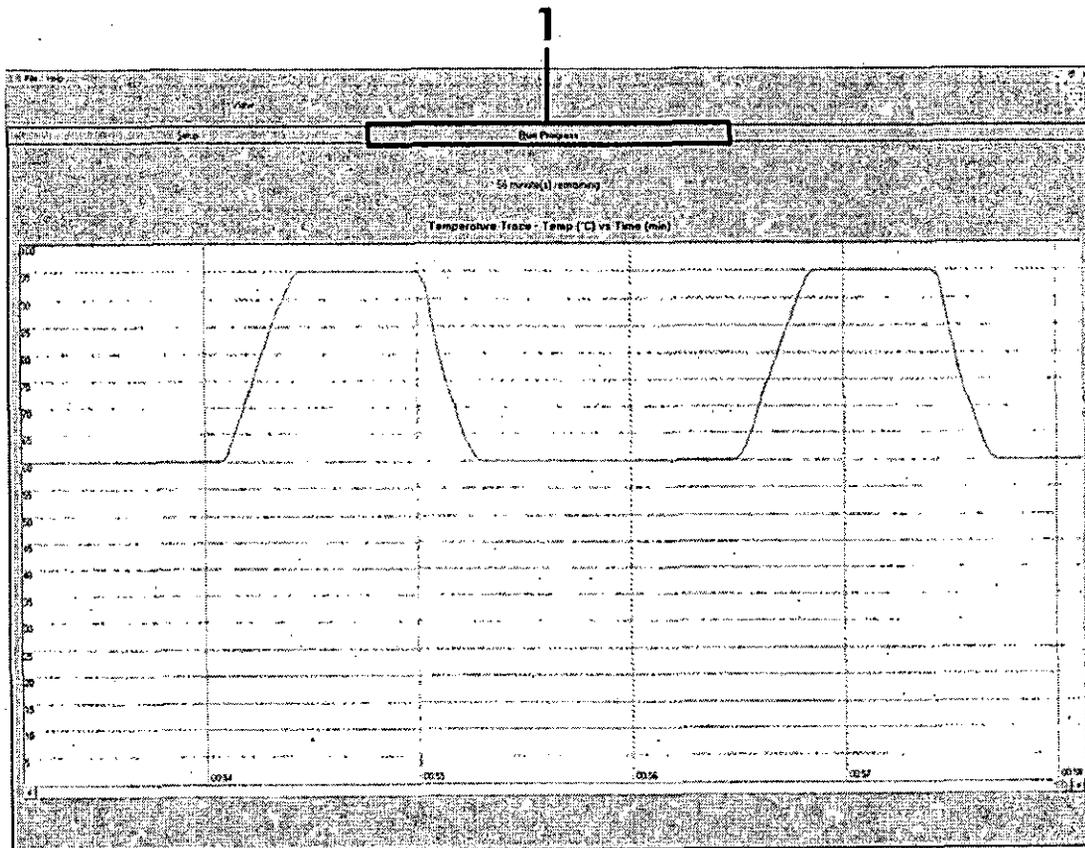


Figure 18. 1 = "Run Progress" tab.

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

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

Note: An explanation of the calculation method is presented in the "Results" section, page 43.

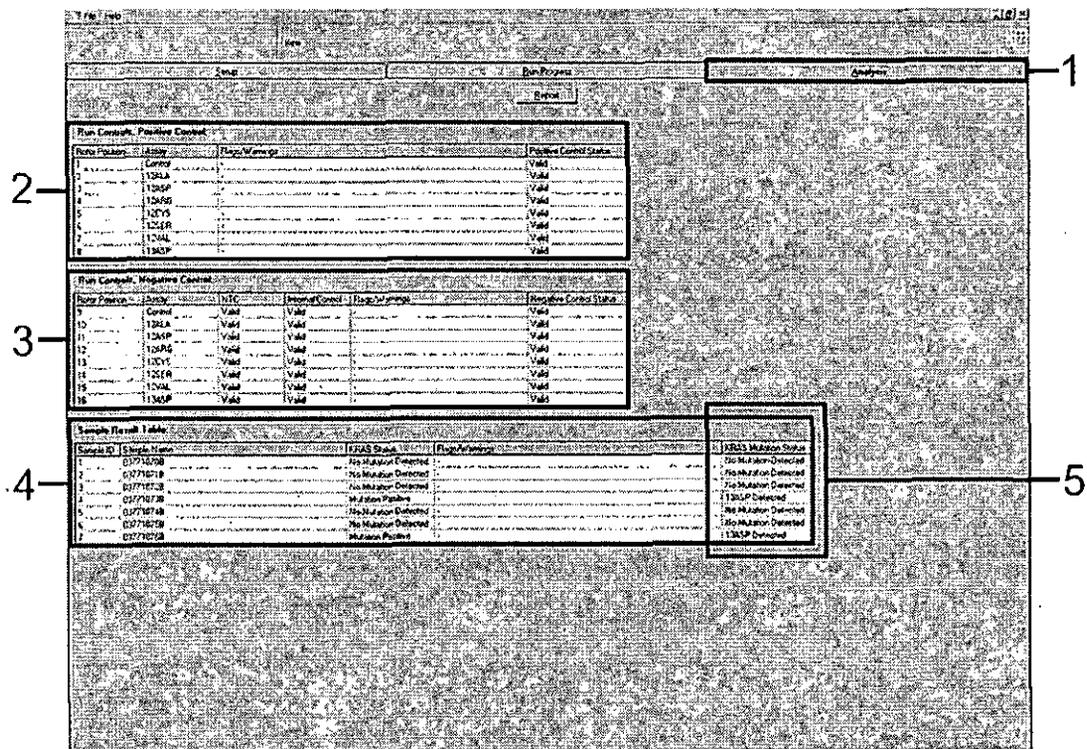


Figure 19. The "Analysis" tab and reporting of results. 1 = "Analysis" tab, 2 = "Run Controls, Positive Control" panel, 3 = "Run Controls, Negative Control" panel, 4 = "Sample Result Table", 5 = "KRAS Mutation Status" column.

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

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

22. Report files can be produced by clicking the "Report" button. The "Report Browser" window will appear. Select "KRAS Analysis Report" under "Templates", and then click the "Show" button (Figure 20).

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

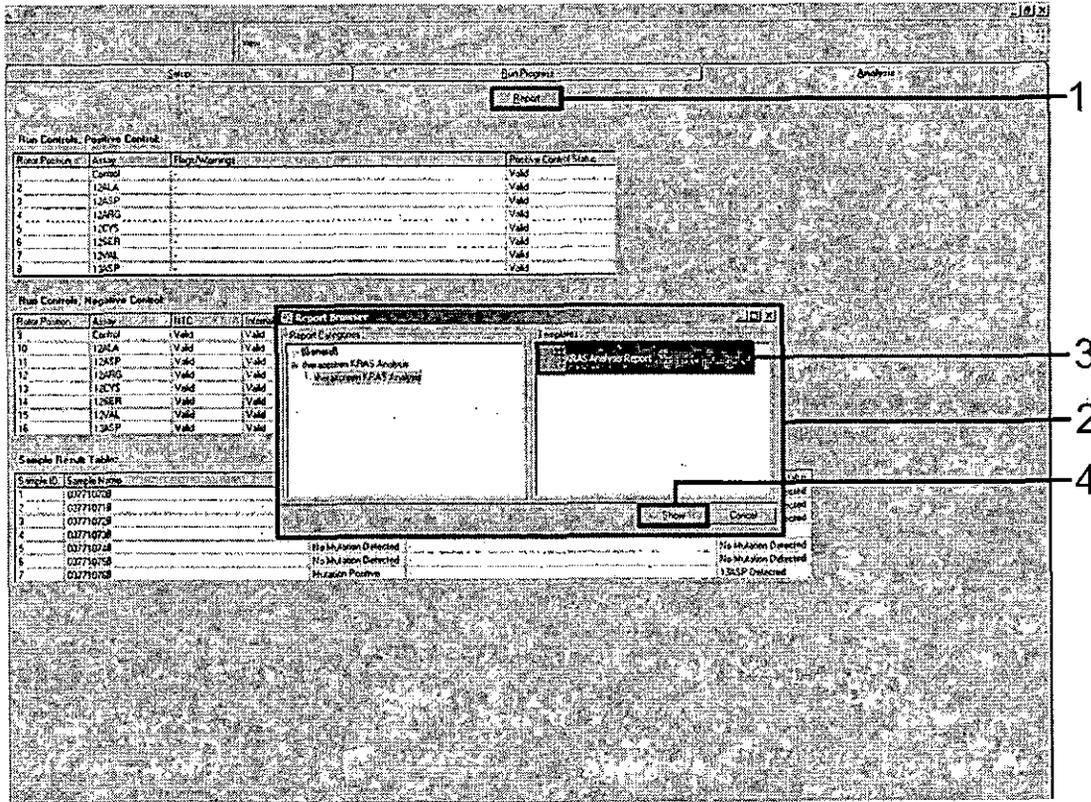


Figure 20. Selecting the "KRAS Analysis Report". 1 = "Report" button, 2 = "Report Browser" window, 3 = "KRAS Analysis Report" selection, 4 = "Show" button.

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.

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 MDx 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 ΔCT value using the equation:

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

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". ΔCT values calculated from background amplification are expected to be greater than the cutoff ΔCT values and the sample will be classed as "No Mutation Detected".

The assay results will be displayed as "Mutation Positive", "No Mutation Detected", "Invalid" or, if a run control fails, "Run Control Failed". For the mutation-positive samples, specific mutations will be reported. Other possible results that may be displayed are discussed in the "Protocol: DNA sample assessment", and "Troubleshooting Guide" sections of this Instructions for Use.

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

Limitations of the Procedure

The test is designed to detect 7 mutation 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 KRAS Kit is not to be used for diagnosis.

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

The *therascreen* KRAS RGQ PCR Kit is only validated for 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.

Specific Performance Characteristics

Analytical performance

The specific performance characteristics of the *therascreen* KRAS RGQ PCR Kit were determined by studies involving formalin-fixed, paraffin-embedded (FFPE) tissue samples collected from colorectal cancer patients (CRC) and 8 formalin-fixed, paraffin-embedded human cell lines (FFPE cell lines) of which 7 harbor known KRAS mutations detected by the assay, and one KRAS wild-type (i.e., no mutations at codon 12 and 13). Mutation status of samples was confirmed by bi-directional Sanger sequencing.

Cut-off

Two hundred twenty FFPE samples were tested using a method following guidance in NCCLS EP17-A (2004) to establish the cut-offs for the assay. 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 10.

Table 10. Established cut-off values for each mutation assay

	Mutant assay (ΔC_T)						
	12ALA	12ASP	12ARG	12CYS	12SER	12VAL	13ASP
Cut-off (ΔC_T) \leq	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 bi-directional 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 (2008). A total of 137 of the FFPE samples returned valid results for both the *therascreen* KRAS RGQ PCR Kit and bi-directional sequencing. The results demonstrated that the KRAS Kit reported two samples as negative, one of which bi-directional sequencing indicated to be positive for 12ASP and the other indicated by bi-directional sequencing to be 13ASP. In contrast, three samples were reported as having a KRAS mutation by the KRAS Kit which was not reported as positive by bi-directional sequencing. In addition, one sample identified as 12ARG by the KRAS Kit was determined to be 12ASP by bi-directional sequencing. There were 5 samples determined to be mutation negative by bi-directional sequencing that were either indeterminate (3 samples) or invalid (2 samples) by the *therascreen*

KRAS RGQ Kit (data not shown). One sample that was indeterminate by Sanger bi-directional sequencing was determined to be 12SER by the KRAS Kit (data not shown).

Table 11 shows the analysis of agreement between *therascreen* KRAS RGQ PCR Kit and bi-directional sequencing.

Table 11 KRAS Kit vs Sanger bi-directional sequencing

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

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

(OPA) of 96.4%. The overall results, excluding 6 failed Sanger samples, are shown in Table 12 below

Table 12. 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

A second unique set of samples was evaluated to supplement the data from the first study. A set of 271 CRC FFPE samples were procured; 250 of unknown mutation status, and 21 samples of known mutation status to enrich for rare mutations were compared to Sanger bi-directional sequencing as described above. A total of 13 (~5%) samples required macrodissection for KRAS Kit evaluation. Out of the 271 samples with bi-directional sequencing results, 24 of the samples were indeterminate by the KRAS Kit (failed control C_T range; data not shown). Concordance analysis was carried out on 247 samples with both valid bi-directional and KRAS Kit results. There were 9 discordant samples. One sample from the 247 samples had a mutation positive result with bi-directional sequencing but a “no mutation detected” result with the KRAS Kit. Eight samples were shown to have a positive result with the KRAS Kit but a negative result with bi-directional sequencing. Overall, agreement was 96.4%. The data supports the accurate performance of the *therascreen* RGQ PCR KRAS Kit.

Table 13 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 14 KRAS Kit vs Sanger bidirectional sequencing (second study)

	Neg	Mutation call by bi-directional sequencing								Total
		12ALA	12ARG	12ASP	12CYS	12SER	12VAL	13ASP		
Negative	132	-	-	-	-	1	-	-	-	133
Positive 12ALA	-	10	-	-	-	-	-	-	-	10
Positive 12ARG	5	-	5	-	-	-	-	-	-	10
Positive 12ASP	-	-	-	31	-	-	-	-	-	31
Positive 12CYS	1	-	-	-	11	-	-	-	-	12
Positive 12SER	-	-	-	-	-	13	-	-	-	13
Positive 12VAL	2	-	-	-	-	-	25	-	-	27
Positive 13ASP	-	-	-	-	-	-	-	-	11	11
Total	140	10	5	31	11	14	25	11	11	247

KRAS Kit call

Limit of detection (LoD)

The working range for the KRAS 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 seven 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; seven with known mutant DNA content and one wild-type were used for this evaluation. The proportion of mutant in total amplifiable DNA (percent mutant DNA) was determined previously using a bi-directional Sanger sequencing method from unfixed cells, followed by relative peak analysis. In the case of three 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 below. Highlighted boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Table 15 Percentage of correct calls

% Correct calls							
% Mutation dilution	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 percent mutation dilutions at both low and high levels within the input range of the assay. Twelve replicates each were evaluated for the high dilution series. 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 16 below. Highlighted boxes indicate the percentage in which greater than 95% of the replicates produced correct calls.

Table 16. Percentage of correct calls (high)

		% 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	33.3	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

* Eleven valid replicates.

Twenty-four replicates were used per dilution for the Low series 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 17 below.

Table 17. Percentage of correct calls (low)

		% Mutation dilution (Low)						
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	9.0	18.0	27.0
	% Correct calls	0	16.7	29.2	58.3	100	100	100
12ARG*		0.33	0.65	1.30	2.60	5.20	10.4	15.6
	% 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	11.7
	% Correct calls	8.3	4.2	20.9	54.2	83.3	100	100
12SER		0.63	1.25	2.50	5.0	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	11.00	16.50
	% Correct calls	4.3	16.7	46.7	75.0	100	100	100
13ASP		0.63	1.25	2.5	5.0	10.0	20.0	30.0
	% Correct calls	0	4.2	8.3	33.3	70.8	100	100

* At 2.60 dilution, the number of valid replicates was 23.

† The number 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 log₂ % 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 18 below.

Table 18. Logistics regression data 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 19 below. At the lower end of the C_T input range, the sensitivity of the assay decreases, as 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 19. LoD values for each mutation assay using FFPE cell lines

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

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 KRAS 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 20 below. For each of the mutations detected by the KRAS 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 KRAS Kit within the prespecified acceptance criteria.

Table 20. Effect of DNA input on ΔC_T values across the input control reaction C_T range — FFPE cell lines

Mutation	Replicate numbers				
	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
	~20-21 C_T	~23-24 C_T	~26-27 C_T	~29-30 C_T	~32-33 C_T
	ΔC_T	ΔC_T	ΔC_T	ΔC_T	ΔC_T
12ALA	1.56	1.25	1.16	1.14	1.27
12ASP*	2.46	2.18	2.11	2.11	1.75
12ARG	1.18	0.63	1.08	0.94	1.06
12VAL	0.29	0.25	0.15	0.26	-0.1
DNA	~22-23 C_T	~24-25 C_T	~27-28 C_T	~29-30 C_T	~32-33 C_T
12SER	2.91	2.21	2.15	2.15	2.08
12CYS	0.98	0.71	0.58	0.81	0.67
13ASP	3.57	2.84	2.54	2.46	2.62

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

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 KRAS 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 21 below.

Table 21. Amplification efficiencies

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	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	
12ASP	Control C _T	20.825	0.083	-1.035	0.01	-1.056	-1.014	0.954	0.056
	12ASP C _T	23.237	0.083	-0.993	0.017	-1.016	-0.97	1.01	
12ARG	Control C _T	20.385	0.13	-1.013	0.16	-1.046	-0.98	0.982	-0.003
	12ARG 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 KRAS 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 KRAS 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, in order 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 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 log₂ 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 $\pm 10\%$.

Table 22. Amplification efficiencies

	Intercept	Intercept standard error	Calculated slope	Standard error slope	Lower two-sided 95% confidence limit (slope)	Upper two-sided 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	0.011	-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 23.

Table 23. Amount of interfering substances tested in each assay (i.e., 1x)

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, 29 samples had necrosis at a level >50% as determined by pathology review. Of these 29 samples, 28 returned valid results that were concordant to bi-directional Sanger sequencing. A single result was invalid due to insufficient DNA.

Cross-contamination

The objective of this study was to determine the extent of cross-contamination, 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 seven 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 non-specific 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, non-specific amplification of KRAS mutations for 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.

Non-specific amplification/cross-reactivity: Wild-type KRAS DNA

In order to address the amount of non-specific amplification of wild-type DNA by reaction mixes designed to amplify specific mutations, sixty (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 was evaluated using the KRAS Kit. For DNA extracted from 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/or lowest ΔC_T values observed for each reaction is shown below.

Table 24. 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 mean (lowest)	Sample 3 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)

Non-specific 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 are presented in the table below and demonstrates 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, however, 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 25 ΔC_T cross-reactivity between mutation reactions using FFPE cell line DNA at the high input range*

	Cut-off	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. 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 two operators at each of 3 sites testing all samples and controls on three lots of *therascreen* KRAS RGQ PCR kits, each day for five days, with two runs per day and with two 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 (one times standard deviation), using C50 and 3xLoD samples are listed in Table 26 and Table 27.

Table 26. Assay variance estimates reproducibility precision estimates

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

Table 27 Repeatability precision estimates

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

The estimated proportion of 3xLoD samples testing mutant and wild-type samples were reported overall and within each of the sites. For all assays and sample combinations, at least 79 out of 80 replicates gave the correct mutation call. The overall proportion of correct calls was 99.6% (1115/1120); 99.6% (558/560) for mutation positive (3xLoD) samples and 99.5% (557/560) for no-mutation detected (wild-type) samples.

Table 28. Correct calls overall

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

* SD=standard deviation.

Sample handling variability

To assess sample handling variability as part of the KRAS Kit Test system 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 one of three 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 three 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 for mutant and wild-type samples showed very close agreement for the results. The results demonstrate the agreement of the DNA extraction procedure and sample processing in conjunction with the KRAS Kit.

Table 29. Comparison by site of mean ΔC_T (SD) values for mutant type samples

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

	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)

Note: "—" denotes a missing value, due to no break-through being observed.

Lot interchangeability

The potential for lot-to-lot variability to impact the mutation detection was assessed. In this study, three 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 formalin-fixed paraffin-embedded 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 KRAS 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 KRAS Kit lots. The ΔC_T values and mutation calls were collected for all test samples. The pre-defined 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 KRAS Kits.

Clinical performance

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 (Erbix). The safety and effectiveness of cetuximab (Erbix) 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 mCRC. Patients were randomized (1:1) to receive either Erbix plus best supportive care (BSC) or BSC alone. Erbix 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.

Note: The KRAS 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-positive” and “No-mutation detected”. In this clinical study 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 KRAS Kit (i.e., the sample may in fact harbor mutations in the KRAS gene not identified by the KRAS Kit such as 13CYS).

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 31 and Figure 21.

Table 31. Overall survival in previously treated EGFR-expressing metastatic colorectal cancer (all randomized and KRAS status)

	All randomized		KRAS Mutation-negative (Wild-type)		KRAS Mutation-positive	
	Erbix plus BSC (N=287)	BSC (N=285)	Erbix plus BSC (N=117)	BSC (N=128)	Erbix plus 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.

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.

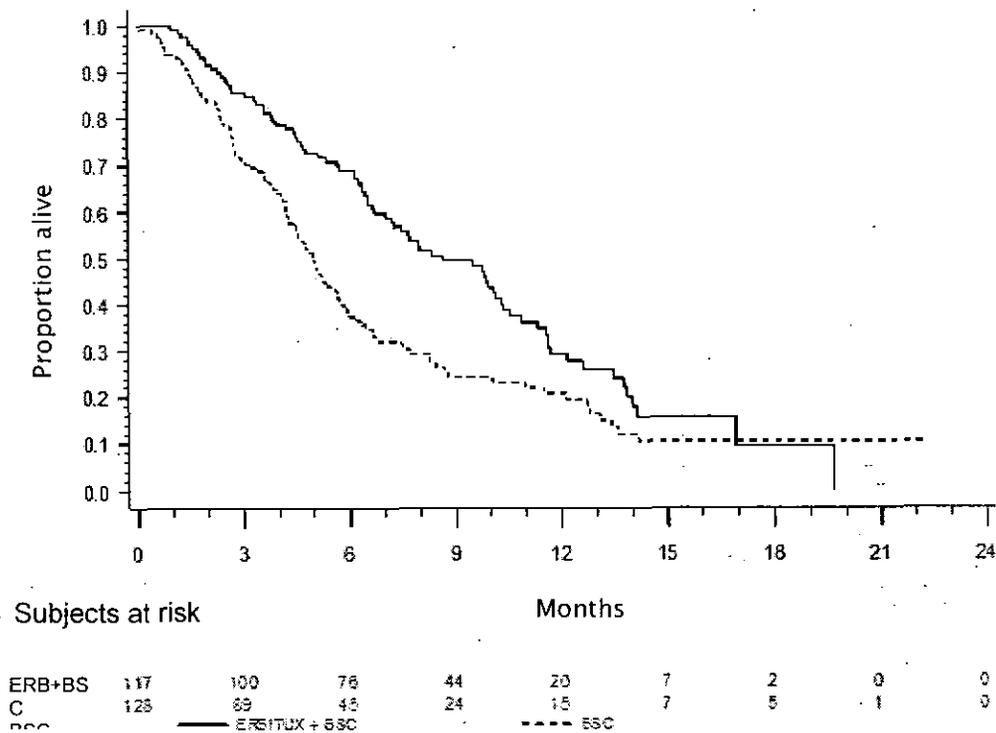


Figure 21. Kaplan–Meier curve for overall survival in patients with KRAS mutation-negative (wild-type) metastatic colorectal cancer.

Refer to the most recent Erbitux drug label available at www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm for more information regarding Erbitux indication.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

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Names and Place of Business of Manufacture



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Troubleshooting Guide

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

Comments and suggestions

Invalid results

- | | |
|--|--|
| a) The storage conditions for one or more components did not comply with the instructions given in "Reagent Storage and Handling", page 13 | 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. |
|--|---|

Rotor-Gene Q *therascreen* KRAS Assay Package flags

Table 33 lists the possible flags that may be generated by the Rotor-Gene Q *therascreen* KRAS Assay Package, their meaning, and actions to be taken.

Table 33. Rotor-Gene Q *therascreen* KRAS Assay Package flags

Flag	Meaning	Action to be taken
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_ASSAY_FAIL	PCR run invalid — FAM C _T out of range for one or more mutation control reactions.	Repeat the entire PCR run.
PC_CTRL_INVALID_DATA	PCR run invalid — Fluorescence data in positive control (Control Reaction Mix) can't be interpreted.	Repeat the entire PCR run.
PC_MUTATION_INVALID_DATA	PCR run invalid — Fluorescence data in positive control (mutation reaction mix) can't be interpreted.	Repeat the entire PCR run.
NTC_INT_CTRL_FAIL	PCR run invalid — Internal control above range for negative control.	Repeat the entire PCR run.

Table continued on next page.

Table 33. Continued.

Flag	Meaning	Action to be taken
NTC_INT_CTRL _EARLY_CT	PCR run invalid — Internal control is below range for negative control.	Repeat the entire PCR run.
NTC_INVALID_CT	PCR run invalid — FAM invalid (smaller than limit) for negative control.	Repeat the entire PCR run.
NTC_INVALID_DATA	PCR run invalid — Fluorescence data in negative control can't be interpreted.	Repeat the entire PCR run.
SAMPLE_CTRL _INVALID_DATA	Sample invalid — Fluorescence data in sample control can't be interpreted.	Setup new PCR run to repeat the relevant sample(s).
SAMPLE_CTRL _HIGH_CONC	Sample Invalid — FAM C _T too low in sample control.	Dilute sample to increase control C _T value. This dilution should be calculated on the assumption that diluting 1:1 with the water supplied in the kit will increase the C _T by 1.0; once sample is diluted, setup new PCR run to repeat sample.
SAMPLE_CTRL_LOW _CONC	Low concentration in sample control (warning, not error).	None.

Table continued on next page.

Table 33. Continued.

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

Table continued on next page.

Table 33. Continued.

Flag	Meaning	Action to be taken
SAMPLE_INT_CTRL _EARLY_CT	Mutation tube invalid — C _T HEX too low for sample (internal control)	If sample given valid status — No action. If sample given invalid status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

Table continued on next page.

Table 33. Continued.

Flag	Meaning	Action to be taken
SAMPLE_INVALID_DATA	Mutation tube invalid — Fluorescence data in internal control can't be interpreted.	If sample given valid status — No action. If sample given invalid status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.
MUTATION_EARLY_CT	Mutation tube invalid — C _T FAM too low for sample.	If sample given valid status — No action. If sample given invalid status, setup new PCR run to repeat sample. If invalid on repeat PCR run, extract the sample from a fresh FFPE section(s). Setup a new PCR run to test fresh extraction. If invalid, repeat this second extraction. If the sample does not give a valid result after this run, the sample is given an indeterminate mutation status and no further testing should be carried out.

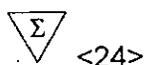
Table continued on next page.

Table 33. Continued.

Flag	Meaning	Action to be taken
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.

Symbols

The following symbols may appear on the packaging and labeling:



Contains reagents sufficient for 24 reactions



Use by



Catalog number



Lot number



Material number (i.e. component, labeling)



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



Contains (contents)



Number (i.e. vials, bottles)

Rn

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



Temperature limitation



Manufacturer



Consult instructions for use



Keep away from sunlight

Contact Information

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

Appendix A: 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. 9021168).

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.

Note: The Rotor-Gene Q *therascreen* KRAS Assay Package only works with Rotor-Gene Q Series Software version 2.1.0 or higher. Make sure the correct version of Rotor-Gene Q MDx software is installed before proceeding with the *therascreen* KRAS Assay Package installation.

Procedure

1. Order the Rotor–Gene Q *therascreen* KRAS RGQ Assay Package CD (QIAGEN, cat. no. 9021168) 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_1.0.12.exe*.
4. The setup wizard will appear. Click “Next” to continue (Figure 21).

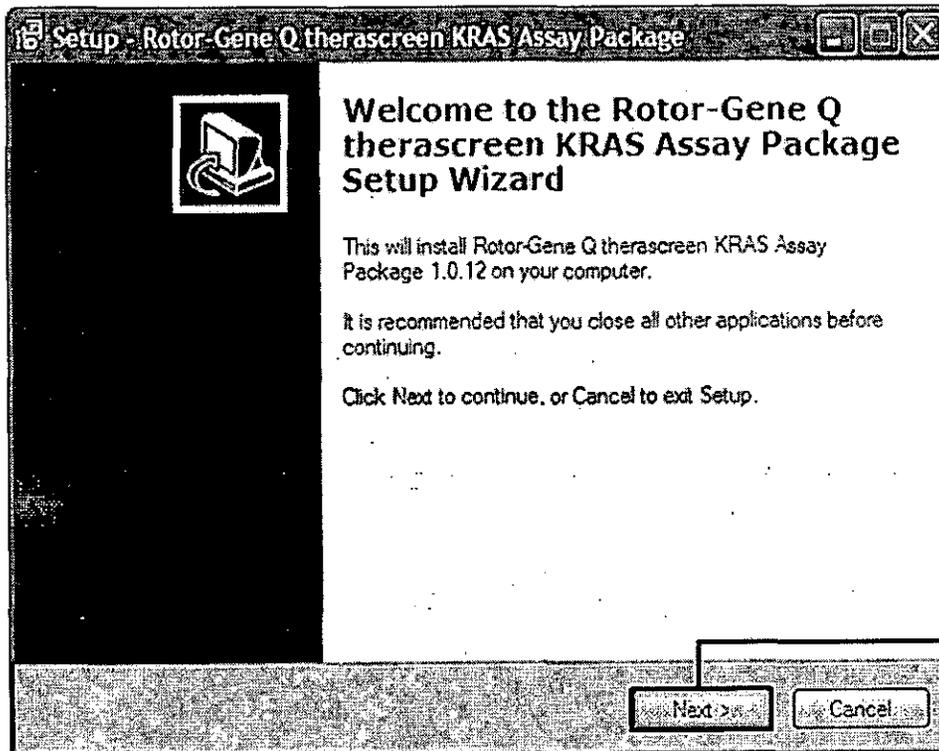


Figure 21. The "Setup" dialog box. 1 = "Next" button.

5. Read the License Agreement in the "License Agreement" dialog box and accept the agreement by checking the statement "I accept the agreement". Click "Next" to continue (Figure 22).

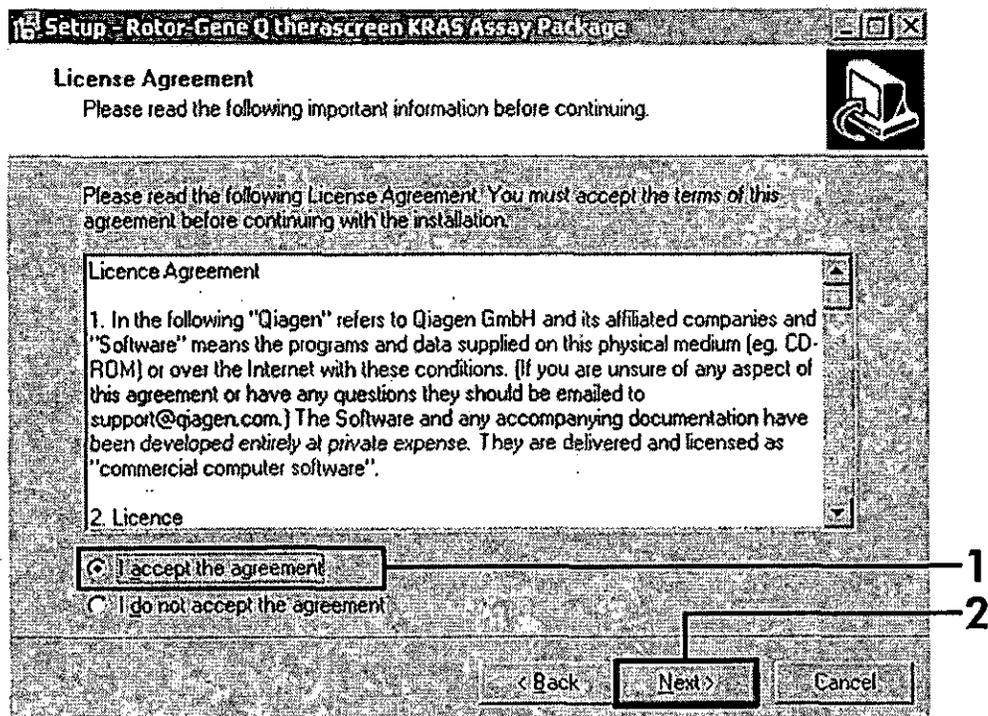


Figure 22. The "License Agreement" dialog box. 1 = "I accept the agreement" statement, 2 = "Next" button.

6. The template setup will start automatically and a final "Setup" dialog box will appear. Click "Finish" to exit the setup wizard (Figure 23).

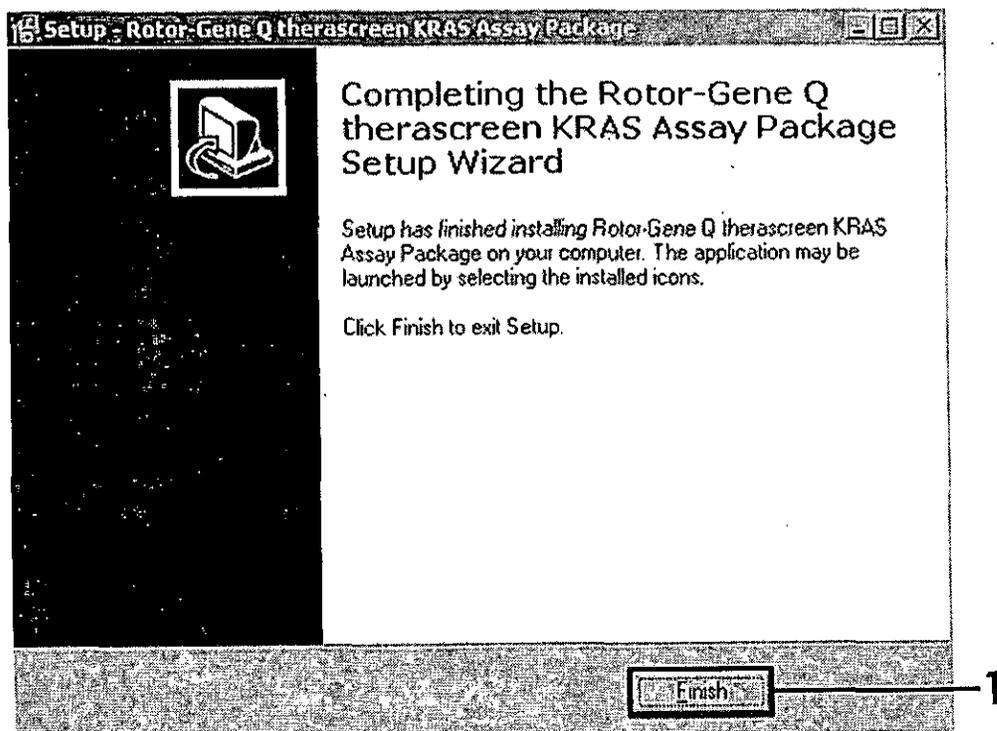


Figure 23. Completing the setup wizard. 1 = "Finish" button.

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

Ordering Information

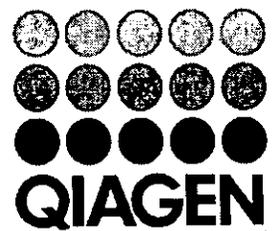
Product	Contents	Cat. no.
<i>therascreen</i> KRAS RGQ PCR KRAS Kit (24)	For 24 reactions: 1 Control Assay, 7 Mutation Assays, Positive Control, Water, <i>Taq</i> DNA Polymerase	870021
Rotor-Gene Q <i>therascreen</i> KRAS Assay Package CD	Software protocol package for use with the <i>therascreen</i> KRAS RGQ PCR KRAS Kit and the QIAGEN Rotor- Gene Q MDx instrument	9021168
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
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

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