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therascreen® RAS Extension Pyro® Kit Handbook

Σ 24

Version 1

IVD

For in vitro diagnostic use

For detection of mutations in exons 3 and 4 of the human KRAS oncogene and exons 2, 3 and 4 of the human NRAS oncogene

CE

REF 971590

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R2 MAT 1085873EN

Sample to Insight

QIAGEN

Contents

Intended Use

The *therascreen* RAS Extension Pyro Kit is an in vitro diagnostic test based on Pyrosequencing® technology for the quantitative detection of mutations in codons 59, 61, 117 and 146 of the human KRAS oncogene and codons 12, 13, 59, 61, 117 and 146 of the human NRAS oncogene, using DNA extracted from formalin-fixed paraffin-embedded (FFPE) metastatic colorectal cancer (mCRC) human tissue.

The *therascreen* RAS Extension Pyro Kit is intended to aid in the identification of mCRC patients more likely to benefit from anti-EGFR therapies such as cetuximab and panitumumab (1).

The *therascreen* RAS Extension Pyro Kit is for use only on the PyroMark Q24 system. PyroMark Q24 systems include the following:

- The PyroMark Q24 instrument or the PyroMark Q24 MDx instrument.
- The PyroMark Q24 Vacuum Workstation or the PyroMark Q24 MDx Vacuum Workstation.
- The PyroMark Q24 Software (version 2.0) or PyroMark Q24 MDx Software (version 2.0).

The *therascreen* RAS Extension Pyro Kit is intended to be used by professional users, such as technicians and physicians who are trained in in vitro diagnostic procedures, molecular biological techniques, and the PyroMark Q24 system.

Summary and Explanation

The *therascreen* RAS Extension Pyro Kit is used for quantitative measurements of mutations in exons 3 and 4 of the human KRAS gene, and exons 2, 3, and 4 of the human NRAS gene. The kit consists of eight assays (Figure 1).

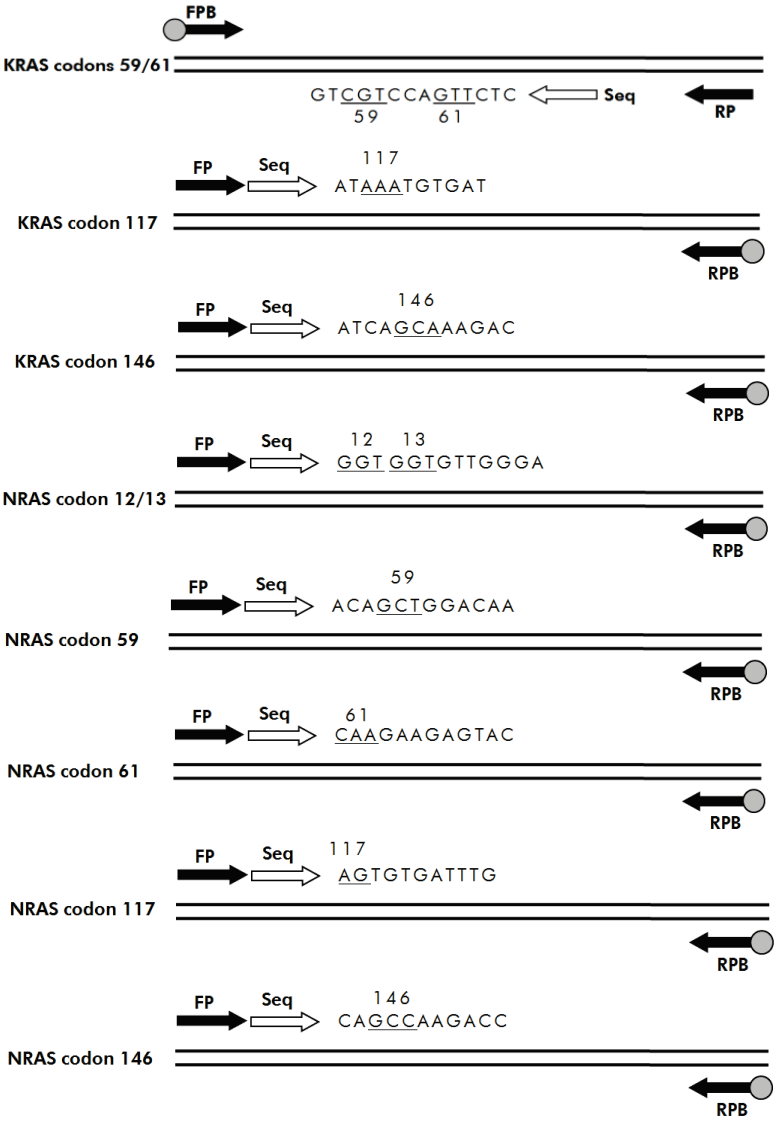


Figure 1. Assays of the *therascreen* RAS Extension Pyro Kit.

The eight regions are amplified separately by PCR, and sequenced through the defined region. Mutations in the covered region will lead to distinct patterns in the Pyrogram® trace that are distinguishable from traces obtained from wild-type samples. Mutations that can be analyzed using the PyroMark® Q24 software are listed in Table 15 (Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays). The assays for KRAS codon 117 and 146, and NRAS codon 12/13, 59, 61, 117 and 146, are sequenced in the forward direction, while the assay for KRAS codon 59/61 is sequenced in the reverse direction. The product consists of a PCR primer mix and a sequencing primer for each assay. The primers are delivered in solution, with each vial containing 24 µl of primer or primer mix.

Principle of the Procedure

Figure 2 (next page) illustrates the workflow of the assay procedure. After a PCR is performed, primers are used to target the region of interest, and the amplicons are immobilized on Streptavidin Sepharose® High Performance beads. Single-stranded DNA is prepared, and the corresponding sequencing primers anneal to the DNA. The samples are then analyzed on the PyroMark Q24 using assay setup files, and a run file.

The "Sequence to Analyze" can be adjusted for detection of different mutations after the run (see "Protocol 6: Analysis of a PyroMark Q24 run", page 33, and "Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays", page 61).

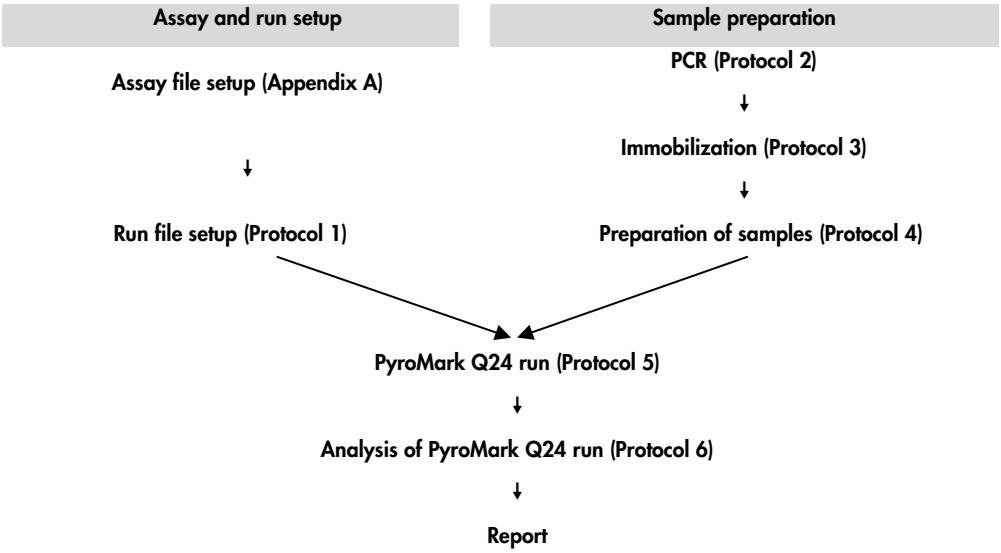


Figure 2. Workflow of *therascreen* RAS Extension Pyro Kit procedure.

Controls

Unmethylated control DNA is included in the kit as a positive control for PCR and sequencing reactions. This control DNA has a wild-type genotype in the sequenced regions using this kit. Include a sample of the control DNA for each assay in every Pyrosequencing run. This is required for adequate result interpretation and identification of low-level mutations (see "Protocol 6: Analysis of a PyroMark Q24 run", page 33).

In addition, a negative control (without template DNA) should be included in every PCR setup for at least one assay.

Materials Provided

Kit contents

Box 1/2

***therascreen* RAS Extension Pyro Kit**	**(24)**
Catalog no.	**971590**
Number of preps	**24**
Seq Primer KRAS 59/61	24 µl
Seq Primer KRAS 117	24 µl
Seq Primer KRAS 146	24 µl
Seq Primer NRAS 12/13	24 µl
Seq Primer NRAS 59	24 µl
Seq Primer NRAS 61	24 µl
Seq Primer NRAS 117	24 µl
Seq Primer NRAS 146	24 µl
PCR Primer KRAS 59/61	24 µl
PCR Primer KRAS 117	24 µl
PCR Primer KRAS 146	24 µl
PCR Primer NRAS 12/13	24 µl
PCR Primer NRAS 59	24 µl
PCR Primer NRAS 61	24 µl
PCR Primer NRAS 117	24 µl
PCR Primer NRAS 146	24 µl
PyroMark PCR Master Mix, 2x	4 x 850 µl
CoralLoad® Concentrate, 10x	1.2 ml
H_2O	6 x 1.9 ml
Unmethylated Control DNA, 10 ng/µl	3 x 100 µl

Box 2/2

Buffers and reagents	Volume
PyroMark Binding Buffer	2 x 10 ml
PyroMark Annealing Buffer	2 x 10 ml
PyroMark Denaturation Solution*	2 x 250 ml
PyroMark Wash Buffer, 10x	2 x 25 ml
Enzyme Mixture	2 vials
Substrate Mixture	2 vials
dATPαS	2 x 1180 µl
dCTP	2 x 1180 µl
dGTP	2 x 1180 µl
dTTP	2 x 1180 µl
therascreen *RAS Extension Pyro Kit Handbook* (English)	1 pc

* Contains sodium hydroxide.

Materials Required but Not Provided

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

Reagents

- DNA isolation kit (see "DNA isolation", page 17)
- Streptavidin Sepharose High Performance (GE Healthcare, cat. no. 17-5113-01; **www.gelifesciences.com**)
- High-purity water (Milli-Q® 18.2 MΩ x cm or equivalent)

  Note: Sufficient water is provided in the kit for PCR, DNA immobilization, and for dissolving the Enzyme Mixture and the Substrate Mixture; additional high-purity water is required to dilute PyroMark Wash Buffer, 10x.

- Ethanol (70%)*

Consumables

- Sterile pipet tips (with filters for PCR setup)
- 24-well PCR plates (see "Recommended 24-well plates", page 12)
- Adhesive foil
- PyroMark Q24 Plate (cat. no. 979301)†
- PyroMark Q24 Cartridge (cat. no. 979302)†

Equipment

- Pipets (adjustable)‡
- Benchtop microcentrifuge‡
- Thermal cycler‡ and appropriate PCR tubes
- PyroMark Q24 MDx or PyroMark Q24 (cat. no. 9001513 or 9001514)‡
- PyroMark Q24 MDx or PyroMark Q24 Vacuum Workstation (cat. no. 9001515 or 9001516 or 9001518 or 9001519)‡
- Plate mixer‡ for immobilization to beads (see "Recommended plate mixers", page 12)
- Heating block‡ capable of attaining 80°C

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

† CE-IVD-marked in accordance with EU Directive 98/79/EC. All other products listed are not CE-IVD-marked based on EU Directive 98/79/EC.

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

Recommended plate mixers

The orbital plate mixers in Table 1 are recommended for use with the *therascreen* RAS Extension Pyro Kit.

Table 1. Plate mixers recommended for use with the *therascreen* RAS Extension Pyro Kit

Manufacturer	Product	Catalog no.
Eppendorf	ThermoMixer® C (basic device)	5382000031
Eppendorf	SmartBlock™ PCR 96, thermoblock for PCR plates 96	5306000006
Thermo Fisher Scientific	Variomag® Teleshake	10448791
Thermo Fisher Scientific	Variomag Monoshake	10515882

Recommended 24-well plates

The 24-well plates in Table 2 are recommended for use with the *therascreen* RAS Extension Pyro Kit.

Table 2. 24-well plates recommended for use with the *therascreen* RAS Extension Pyro Kit

Manufacturer	Product	Catalog no.
Thermo Fisher Scientific	Thermo-Fast PCR Plate, 24-well	AB0624
Corning	Axygen® 24 Well Polypropylene PCR Microplate	PCR-24-C
4titude	FrameStar® Break-a-way 96 wells, clear tubes	4ti-1000
Kisker	Quali – PCR Plates without frame	G030

Warnings and Precautions

For in vitro diagnostic use

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

General precautions

Always pay attention to the following:

- The components of this product are sufficient to perform 24 reactions for each assay.
- Use sterile pipet tips (with filters for PCR setup).
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.
- Failed results are not a basis for judgment of mutational status.

Reagent Storage and Handling

The *therascreen* RAS Extension Pyro Kit is shipped in two boxes. The *therascreen* RAS Extension Pyro Kit (box 1/2) is shipped on dry ice. PyroMark PCR Master Mix, CoralLoad Concentrate, unmethylated control DNA, and all primers should be stored at –15 to –25°C upon arrival.

The Pyro Buffers and Reagents (box 2/2) containing Buffers, Enzyme Mixture, Substrate Mixture, dATPαS, dCTP, dGTP, and dTTP (the reagents for Pyrosequencing analysis) is shipped on cool packs. These components should be stored at 2–8°C upon arrival. To minimize loss of activity, it is advisable to keep both the enzyme mixture, and the substrate mixture in the vials supplied.

Reconstituted enzyme and substrate mixtures are stable for at least 10 days at 2–8°C. Reconstituted enzyme and substrate mixtures can be frozen, and stored in their vials at –15 to –25°C. Frozen reagents should not be subjected to more than six freeze–thaw cycles.

Note: Nucleotides should not be frozen.

When stored under these conditions, the *therascreen* RAS Extension Pyro Kit is stable until the kit expiration date.

Specimen Collection, Preparation for Analysis, and Storage

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

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

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

Preparation of tissue samples

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

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

Preparation of tissue samples for DNA extraction

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

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

Storage

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

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

Procedure

DNA isolation

The QIAGEN kit shown in Table 3 is recommended for DNA purification for the indicated human sample type, as well as for use with the *therascreen* RAS Extension Pyro Kit. To use this kit, follow the DNA purification instructions in the respective kit handbook.

Table 3. DNA purification kit recommended for use with the *therascreen* RAS Extension Pyro Kit

Sample type	Nucleic acid isolation kit	Catalog number (QIAGEN)
Paraffin-embedded tissue	QIAamp® DNA FFPE Tissue Kit (50)	56404

Protocol 1: Run setup for the PyroMark Q24 system

Things to do before starting

- Create an Assay Setup as described in "Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays" on page 61. This must only be set up once, before running the RAS Extension Pyro assay for the first time.
- Avoid placing samples with high signal intensities next to "no template control" wells, and wells with expected low signals. This may lead to cross talk signals between wells, where a signal from one well is detected in a neighboring well.

Procedure

1. Click  in the toolbar.

   A new run file is created.
2. Enter the run parameters (see "Run parameters", page 18).
3. Set up the plate by adding assays for all eight assays of the *therascreen* RAS Extension Pyro Kit, to wells corresponding to the samples to analyze.

Note: A negative control sample (without template DNA) must be included in every PCR setup for at least one assay.

Note: Include a sample with unmethylated control DNA as a wild-type control for each assay in every Pyrosequencing run (see Figure 2, page 8).

4. When the run is set up and ready to run on the PyroMark Q24 system, print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Select "Pre Run Information" from the "Tools" menu. When the report appears, click  .
5. Close the run file and copy it to a USB stick (supplied with the system) using Windows® Explorer.

   Note: The printed pre-run Information can be used as a template for the sample setup (see "Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads", page 23).

   Note: To run the plate on the PyroMark Q24 system, see "Protocol 5: Running the PyroMark Q24", page 30.

Run parameters

- **Run name**: The name of the run is given when the file is saved. Renaming the file also changes the name of the run.
- **Instrument method**: Select the instrument method according to the cartridge that will be used for the run; see the instructions supplied with the products.
- **Plate ID (optional)**: Enter ID of the PyroMark Q24 Plate.
- **Bar code (optional)**: Enter a bar code number for the plate or, if you have a bar code reader connected to your computer, place the mouse cursor in the "Barcode" text box (by clicking the box) and scan the bar code.
- **Kit and reagent ID (optional)**: Enter the lot number for the *therascreen* RAS Extension Pyro Kit to be used. The lot number can be found on the product label.

  Note: We recommend entering both lot numbers so that any unexpected problems with the *therascreen* RAS Extension Pyro Kit can be traced.

- **Run note (optional)**: Enter a note about the contents or purpose of the run.

Add assay files

To add an assay to a well, you can either:

- Right-click the well and select "Load Assay" from the context menu.
- Select the assay in the shortcut browser, and click and drag the assay to the well.

A well is color-coded according to the assay loaded to the well.

Enter sample IDs and notes

To enter a sample ID or note, select the cell and enter the text.

To edit a sample ID or note, either select the cell (the current contents will be selected) or double-click the cell.

Protocol 2: PCR using the PCR reagents supplied with the *therascreen* RAS Extension Pyro Kit

This protocol is for PCR amplification of eight separate regions in exons 3 and 4 of the human KRAS gene, and exons 2, 3, and 4 of the human NRAS gene using the *therascreen* RAS Extension Pyro Kit.

Important points before starting

- The HotStarTaq® DNA polymerase in the PyroMark PCR Master Mix requires an activation step of 15 minutes at 95°C.
- Set up all reaction mixtures in an area separate from that used for DNA purification, adding template to the PCR, PCR product analysis, or preparation of samples prior to Pyrosequencing analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

Things to do before starting

- Before opening the tubes with PCR primers, centrifuge briefly to collect contents at the bottom of the tubes.
- Adjust the concentration of the control and sample DNA, if necessary, to 0.4–2 ng/µl.

Procedure

1. Thaw all necessary components (Table 4).

   Mix well before use.

2. Prepare a reaction mix for each PCR primer set according to Table 4.

   The reaction mix typically contains all of the components needed for PCR except the sample.

   Prepare a volume of reaction mix greater than that required for the total number of PCR assays to be performed.

Table 4. Preparation of reaction mix for each PCR primer mix

Component	Volume/reaction (µl)
PyroMark PCR Master Mix, 2x	12.5
CoralLoad Concentrate, 10x	2.5
PCR Primer KRAS 59/61 **or** PCR Primer KRAS 117 **or** PCR Primer KRAS 146 **or** PCR Primer NRAS 12/13 **or** PCR Primer NRAS 59 **or** PCR Primer NRAS 61 **or** PCR Primer NRAS 117 **or** PCR Primer NRAS 146	1
Water (H_2O, supplied)	4
Total volume	**20**

3. Mix the reaction mix thoroughly, and dispense 20 µl into each PCR tube.

   It is not necessary to keep PCR tubes on ice since HotStarTaq DNA polymerase is inactive at room temperature.

4. Add 5 µl template DNA (2–10 ng of genomic DNA) to the individual PCR tubes (Table 5), and mix thoroughly.

   Note: A negative control sample (without template DNA) should be included in every PCR setup for at least one assay.

   Note: Include a sample with unmethylated control DNA as a wild-type control for each assay in every Pyrosequencing run (see "Controls", page 8).

Table 5. Preparation of PCR

Component	Volume/reaction (µl)
Reaction mix	20
Sample DNA	5
Total volume	**25**

5. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 6.

Table 6. Optimized cycling protocol

	Time	Temperature	Comments
Initial activation step:	15 min	95°C	HotStarTaq DNA polymerase is activated by this heating step
3-step cycling:			
Denaturation	20 s	95°C	
Annealing	30 s	53°C	
Extension	20 s	72°C	
Number of cycles	42	–	
Final extension:	5 min	72°C	

6. Place the PCR tubes in the thermal cycler and start the cycling program.
7. After amplification, proceed with "Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads", page 23.

   The PCR samples can be stored at 2–8°C for up to 3 days.

Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads

This protocol is for immobilization of template DNA to Streptavidin Sepharose High Performance prior to analysis on the PyroMark Q24 system.

Things to do before starting

- Allow all required reagents and solutions to reach room temperature (15–25°C) before starting.
- Switch on the PyroMark Q24 at least 30 minutes before starting a run. The power switch is located at the rear of the instrument.
- Place one PyroMark Q24 plate holder on a preheated heating block at 80°C. Leave a second PyroMark Q24 plate holder at room temperature (15–25°C).
- PyroMark Wash Buffer is supplied as a 10x concentrate. Before using for the first time, dilute to a 1x working solution by adding 225 ml high-purity water to 25 ml 10x PyroMark Wash Buffer (final volume of 250 ml).

  Note: The 1x PyroMark Wash Buffer working solution is stable at 2–8°C until the marked expiration date.
- Prepare the PyroMark Q24 Vacuum Workstation for sample preparation as described in the *PyroMark Q24 User Manual*.

Procedure

1. Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.
2. Prepare a master mix for DNA immobilization according to Table 7.

   Prepare a volume greater than that required for the total number of reactions to be performed (for the number of reactions + one extra).

Table 7. Master mix for DNA immobilization

Component	Volume/reaction (µl)
PyroMark Binding Buffer	40
Water (H_2O, supplied)	29
Streptavidin Sepharose High Performance	1
Total volume	**70**

3. Add 70 µl of the master mix to wells of a 24-well PCR plate as predefined in the run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 17).

   Sepharose beads sediment quickly. Ensure the homogeneity of the master mix by frequent mixing using a pipet or pulse vortexing. Do not centrifuge the master mix.

4. Add 10 µl of biotinylated PCR product from Protocol 2 to each well containing master mix as predefined in the run setup (see "Protocol 2: PCR using the PCR reagents supplied with the *therascreen* RAS Extension Pyro Kit", page 20).

   The total volume per well should be 80 µl after addition of the master mix and PCR product.

5. Seal the PCR plate using adhesive foil.

   Ensure that no leakage is possible between the wells.

6. Agitate the PCR plate at room temperature (15–25°C) for 5–10 minutes at 1400 rpm.

   During this step, proceed immediately with "Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24", page 25.

Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24

This protocol is for preparation of single-stranded DNA, and annealing of the sequencing primer to the template prior to Pyrosequencing analysis on the PyroMark Q24.

Important points before starting

- Before opening the tubes with sequencing primers, centrifuge briefly to collect contents at the bottom of the tubes.
- Add the different sequencing primers in the same pattern as predefined for the plate in the run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 17), depending on the region of analysis.
- Perform the function test for the filter probes as described in the *PyroMark Q24 User* Manual on a regular basis and exchange filter probes when indicated.

Procedure

1. Dilute a sufficient amount of each sequencing primer in PyroMark Annealing Buffer as shown in Table 8.

   Prepare a volume of diluted sequencing primer greater than that required for the total number of samples to be sequenced (for the number of samples plus one extra).

   Do not dilute and store more sequencing primer than is needed.

Table 8. Example of dilution of the sequencing primers

Component	Volume/sample (µl)	Volume for 9 + 1 reactions (µl)
PyroMark Annealing Buffer	24.2	242
Seq Primer KRAS 59/61 **or** Seq Primer KRAS 117 **or** Seq Primer KRAS 146 **or** Seq Primer NRAS 12/13 **or** Seq Primer NRAS 59 **or** Seq Primer NRAS 61 **or** Seq Primer NRAS 117 **or** Seq Primer NRAS 146	0.8	8
Total volume	**25**	**250**

2. Add 25 µl of diluted sequencing primer to each well of the PyroMark Q24 Plate, according to the run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 17).

   Keep one of the PyroMark Q24 plate holders (supplied with the PyroMark Q24 Vacuum Workstation) at room temperature (15–25°C), and use it as support when preparing and moving the plate.

3. Switch on the vacuum pump of the PyroMark Q24 Vacuum Workstation.
4. Place the PCR plate from Protocol 3, and the PyroMark Q24 Plate on the vacuum workstation (Figure 3).

   Inspect the PCR plate and ensure the Sepharose beads are in solution. Ensure that the PCR plate is in the same orientation as when samples were loaded.

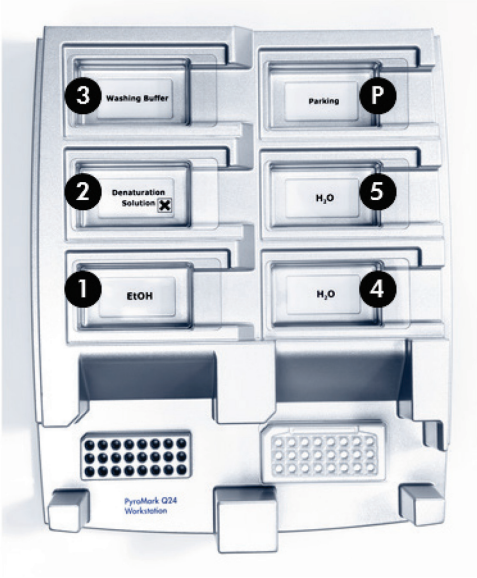


Figure 3. Placement of PCR plate and PyroMark Q24 plate on the vacuum workstation.

5. Apply vacuum to the tool by switching the vacuum on.
6. Slowly lower the filter probes of the vacuum tool into the PCR plate to capture the beads containing immobilized template. Hold the probes in place for 15 s. Practice care while lifting the vacuum tool.

   Note: Sepharose beads sediment quickly. Capturing the beads must take place immediately following agitation. If more than 1 min has elapsed since the plate was agitated, agitate the plate again for 1 min before capturing the beads.

   Inspect the PCR plate for complete uptake of all samples by the vacuum tool.
7. Transfer the vacuum tool to the trough containing 40 ml of 70% ethanol (**trough 1; Figure 3**). Flush the filter probes for 5 s.
8. Transfer the vacuum tool to the trough containing 40 ml of Denaturation Solution (**trough 2; Figure 3**). Flush the filter probes for 5 s.

9. Transfer the vacuum tool to the trough containing 50 ml Wash Buffer (**trough 3; Figure 3**). Flush the filter probes for 10 s.
10. Raise the vacuum tool up and backwards, over a 90° vertical angle, for 5 s to drain liquid from the filter probes (Figure 4).

Figure 4. Illustration of the vacuum tool raised to beyond 90° vertical angle.

11. While the vacuum tool is held over the PyroMark Q24 Plate, turn the vacuum off.
12. Release the beads into the PyroMark Q24 Plate by lowering the filter probes into the diluted sequencing primer, and moving the vacuum tool gently from side to side.

    Note: Be careful not to damage the surface of the PyroMark Q24 Plate by scratching it with the filter probes.
13. Transfer the vacuum tool to the trough containing high-purity water (**trough 4; Figure 3**), and agitate the vacuum tool for 10 s.
14. Wash the filter probes by lowering the probes into the high-purity water trough (**trough 5; Figure 3**), and apply the vacuum. Flush the probes with 70 ml of high-purity water.
15. Raise the vacuum tool up and backwards, over a 90° vertical angle, for 5 s to drain liquid from the filter probes (Figure 4).
16. Switch off the vacuum tool, and place the vacuum tool in the Parking (P) position.

17. Switch off the vacuum pump.

    Note: At the end of a workday, liquid waste and remaining solutions should be discarded, and the PyroMark Q24 Vacuum Workstation should be inspected for dust and spills. See “Appendix B: Emptying the Waste Container and Troughs”, page 66.

18. Heat the PyroMark Q24 Plate with the samples at 80°C for 2 min using the pre-warmed PyroMark Q24 plate holder.

19. Remove the PyroMark Q24 Plate from the hot plate holder, and place it on a second PyroMark Q24 plate holder that was kept at room temperature (15–25°C), to allow samples cool to room temperature for 10–15 min.

    Proceed directly with “Protocol 5: Running the PyroMark Q24”, page 30.

Protocol 5: Running the PyroMark Q24

This protocol describes the preparation and loading of PyroMark Gold Q24 Reagents, into the PyroMark Q24 Cartridge, and starting and finishing a run on the PyroMark Q24. For a detailed description about how to set up a run, see the *PyroMark Q24 User Manual*.

Important points before starting

- The pre-run information report, found in the "Tools" menu at run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 17), provides information about the volume of nucleotides, enzyme, and substrate buffer needed for a specific run.
- Load the cartridge with disposable tips (without hydrophobic filters), to ensure correct functioning of the cartridge.

Procedure

1. Dissolve the freeze-dried enzyme and substrate mixtures in 620 µl water (H_2O, supplied).
2. Mix by swirling the vial gently.

   Note: Do not vortex!

   To ensure that the mixture is fully dissolved, leave the mixture at room temperature (15–25°C) for 5–10 min. Make sure the solution is not turbid before filling the PyroMark Q24 Cartridge. If the reagents are not to be used immediately, place the reagent vials on ice, or in a refrigerator.
3. Allow the reagents and the PyroMark Q24 Cartridge to reach ambient temperature (20–25°C).
4. Place the PyroMark Q24 Cartridge with the label facing toward you.
5. Load the PyroMark Q24 Cartridge with the appropriate volumes of nucleotides, enzyme, and substrate mixes according to Figure 5.

   Make sure that no air bubbles are transferred from the pipet to the cartridge.

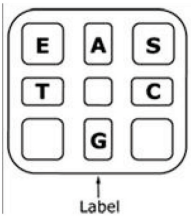


Figure 5. Illustration of the PyroMark Q24 Cartridge as seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (**E**), substrate mixture (**S**), and nucleotides (**A**, **T**, **C**, **G**) according to the volume information given in the Pre Run information report, found in the "Tools" menu at run setup.

6. Open the cartridge gate and insert the filled reagent cartridge with the label facing out. Push the cartridge in fully, and then push it down.
7. Make sure the line is visible in front of the cartridge and close the gate.
8. Open the plate-holding frame and place the plate on the heating block.
9. Close the plate-holding frame and the instrument lid.
10. Insert the USB stick (containing the run file) into the USB port at the front of the instrument.

    Do not remove the USB stick before the run is finished.
11. Select "Run" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
12. Select the run file using the ▲ and ▼ screen buttons.

    To view the contents of a folder, select the folder and press "Select". To return to the previous view, press "Back".
13. When the run file is selected, press "Select" to start the run.
14. When the run is finished and the instrument confirms that the run file has been saved to the USB stick, press "Close".
15. Remove the USB stick.
16. Open the instrument lid.
17. Open the cartridge gate and remove the reagent cartridge by lifting it up and pulling it out.
18. Close the gate.

19. Open the plate-holding frame and remove the plate from the heating block.
20. Close the plate-holding frame and the instrument lid.
21. Discard the plate and clean the cartridge, as per the instructions in the product sheet supplied with the cartridge.
22. Analyze the run according to “Protocol 6: Analysis of a PyroMark Q24 run”, page 33.

Protocol 6: Analysis of a PyroMark Q24 run

This protocol describes the mutation analysis of a finished *therascreen* RAS Extension Pyro run using PyroMark Q24 Software.

Procedure

1. Insert the USB stick containing the processed run file into the computer's USB port.
2. Move the run file from the USB stick to the desired location on the computer using Windows Explorer.
3. Open the run file in the AQ mode of PyroMark Q24 Software either by selecting "Open" in the "File" menu or by double-clicking the file ( ) in the shortcut browser.
4. Using the RAS Extension Plug-In Report to generate a Plug-in report, select "AQ Add On Reports/RAS Extension" from "Reports" in the menu (Figure 6).

   Note: Mutations in KRAS codon 61must in addition, be analyzed with the separate KRAS plug-in by selecting "AQ Add On Reports/KRAS/Codon 61" from "Reports" in the menu.

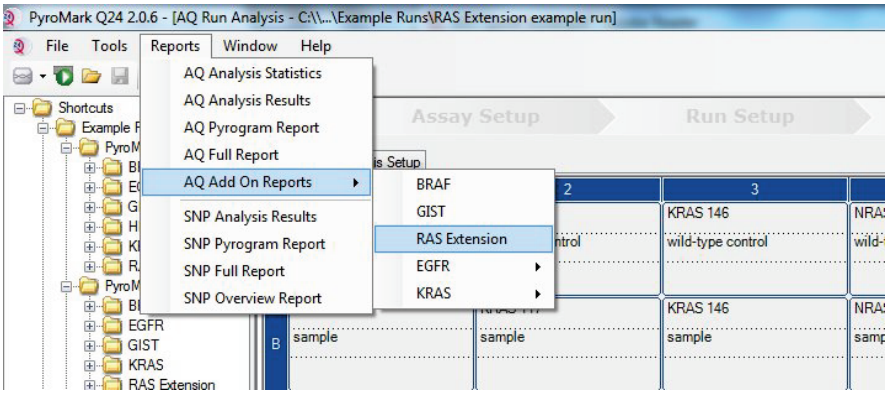


Figure 6. RAS Extension Plug-In Report menu.

The wells will automatically be analyzed for all mutations for which LOD is given in Table 9, page 40. The results will be presented in an overview table (Figure 7), followed by the detailed results which include Pyrograms and analysis quality.

Summary

Well	Assay Name	Sample ID	Result	Frequency [% units]	Nucleotide Substitution	Amino Acid Substitution	Info
A1	KRAS Codon 59	wild-type control	No mutation detected				
A2	KRAS Codon 117	wild-type control	No mutation detected				
A3	KRAS Codon 146	wild-type control	No mutation detected				
A4	NRAS Codon 12 and 13	wild-type control	No mutation detected				
A5	NRAS Codon 59	wild-type control	No mutation detected				
A6	NRAS Codon 61	wild-type control	No mutation detected				
A7	NRAS Codon 117	wild-type control	No mutation detected				
A8	NRAS Codon 146	wild-type control	No mutation detected				
B1	KRAS Codon 59	sample	Mutation	35,0	175G>A	A59T	
B2	KRAS Codon 117	sample	No mutation detected				
B3	KRAS Codon 146	sample	Mutation	29,6	437C>T	A146V	
B4	NRAS Codon 12 and 13	sample	No mutation detected				
B5	NRAS Codon 59	sample	Mutation	20,5	176C>G	A59G	
B6	NRAS Codon 61	sample	No mutation detected				
B7	NRAS Codon 117	sample	Potential low level mutation	5,0	351G>C	K117N	⚠
B8	NRAS Codon 146	sample	No mutation detected				
C1	KRAS Codon 59	NTC	Failed Analysis				⚠
C2	KRAS Codon 117	NTC	Failed Analysis				⚠
C3	KRAS Codon 146	NTC	Failed Analysis				⚠
C4	NRAS Codon 12 and 13	NTC	Failed Analysis				⚠
C5	NRAS Codon 59	NTC	Failed Analysis				⚠
C6	NRAS Codon 61	NTC	Failed Analysis				⚠
C7	NRAS Codon 117	NTC	Failed Analysis				⚠
C8	NRAS Codon 146	NTC	Failed Analysis				⚠

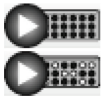
⚠ See detailed results below.

NOTE: The result must be validated by comparing the observed peaks with the expected peak heights displayed as grey bars. For further information about data evaluation and result interpretation please refer to the handbook.

Figure 7. RAS Extension Plug-In Report.

5. Using AQ analysis:

To analyze the run and get an overview of the results click one of the "Analyze" buttons.



Analyze all wells.

Analyze the selected well.

The analysis results (allele frequencies) and quality assessment are displayed above the variable position in the Pyrogram trace. For more details on how to analyze a run, see the *PyroMark Q24 User Manual*.

To generate a report, select "AQ Full Report" or "AQ Analysis Results" in the "Reports" menu.

Note: For reliable results, we recommend single peak heights above 30 RLU. Set 30 RLU as the "required peak height for passed quality" in assay setup and ensure that the A-peak reduction factor is set to 0.86 for analysis of NRAS codon 61 (see Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays", page 61, and the *PyroMark Q24 User Manual*).

The "AQ Analysis Results" report should be used for documentation and interpretation of allele quantification. The numbers shown in the Pyrogram are rounded and do not show the exact quantification.

Note: The Pyrogram should always be compared to the histogram, which can be displayed by right clicking in the Pyrogram window. The measured peaks should match the height of the histogram bars. See also "Interpretation of Results", page 37.

Re-analysis of samples with no mutation detected with the standard "Sequence to analyze" or with "Check" or "Failed" quality assessment.

The standard "Sequence to Analyze" as defined in the Analysis Setup, addresses the most frequent point mutations in the *therascreen* RAS Extension Pyro assays.

We strongly recommend manually reanalyzing all samples with no mutation detected with the standard "Sequence to Analyze", as well as samples which received "Check" or "Failed" quality assessment. "Check" and "Failed" quality assessments may indicate a mutation that is not addressed by the standard "Sequence to Analyze", resulting in unexpected reference peaks.

To reanalyze and target other mutations, go to "Analysis Setup", and change to "Sequence to Analyze" to variants described in Table 16 and Table 17 in Appendix A or variants for other rare or unexpected mutations. Click "Apply", and then click "To All" when the "Apply Analysis Setup" window appears.

Updated frequencies of mutations in the human KRAS and NRAS genes are provided online by the Sanger Institute at **www.sanger.ac.uk/genetics/CGP/cosmic/**.

Note: After changing "Sequence to Analyze", ensure that the threshold for single peak height is set to 30 RLU, and ensure that the A-peak reduction factor is set to 0.86 for analysis of NRAS codon 61 (see "Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays", page 61).

Note: Additional rare or unexpected mutations may be present in the sequenced region and can be analyzed using alternative "Sequence to Analyze", considering unexpected mutations.

Note: In case the measured peaks do not match the height of the histogram bars and cannot be explained by rare or unexpected mutations, the result is not a basis for judgment of mutational status. It is recommended to rerun the sample.

Interpretation of Results

Interpretation of analysis results and detection of low-level mutations

Include a sample of the control DNA for each assay in every Pyrosequencing run. This is required for adequate result interpretation and identification of low-level mutations, and as a control for background levels. The measured frequency of the control sample should be smaller or equal to the limit of blank (LOB). The LOB (limit of blank) and LOD (limit of detection) values given in the handbooks can be used when determining the presence of a mutation. These values were obtained using plasmid mixtures carrying the wild-type or the relevant mutated sequence.

After analysis using PyroMark Q24 Software or the Plug-In Reports, three results are possible. For LOD data, see Table 9.

- Mutation frequency < LOD: Mutation not detected
- Mutation frequency > LOD + 3 % units: Mutation
- Mutation frequency ≥ LOD and ≤ LOD + 3 % units: Potential low-level mutation

Note: If using the RAS Extension Plug-In Report (see step 5 of "Protocol 6: Analysis of a PyroMark Q24 run", page 33) and this occurs, a warning will be issued.

The range from LOD to LOD + 3 % units allows for sensitive detection of low-level mutations under optimal conditions. A measured frequency above LOB in the unmethylated control sample indicates a higher than usual level of background in the respective run, which may impact allele quantification, especially for low mutational levels. Therefore results with the "Potential low level mutation" warning must be carefully evaluated.

Samples with a reported potential low-level mutation should only be considered positive for the mutation, if confirmed by re-running in duplicate together with the unmethylated control DNA. The result of both duplicates should report the same mutation with values ≥ LOD, and the control sample should report as "No mutation detected". Otherwise the sample should be judged as "No mutation detected".

Increased background for a mutation can be detected by comparing the LOB values listed in the handbook, to the measurements obtained with the unmethylated control DNA. Samples with a reported potential low-level mutation can be rated as "Mutation not detected" without repetition if the measured frequency for the unmethlyated control DNA is higher than the LOB value listed in the handbook for the relevant mutation. Therefore three different scenarios are possible with reported potential low level mutations.

1. Measurement frequency with unmethylated control DNA >LOB for this mutation: Sample can be rated as "Mutation not detected" without repetition.
2. Result not reproduced in duplicate with the same result: Rate sample as "Mutation not detected".
3. Same results reproduced in duplicate and unmethylated control DNA <LOB for relevant mutation: Mutation detected.

Note: The Pyrogram should always be compared to the histogram, which can be displayed by right-clicking in the Pyrogram window. The measured peaks should match the height of the histogram bars. The Pyrograms should be examined for the appearance of unexpected peaks. If the measured peaks do not match the height of the histogram bars and cannot be explained by rare or unexpected mutations, it is recommended to rerun the sample. The failed result is not a basis for judgment of mutational status. For a valid mutation, a change in the peak height is always related to a corresponding change in height of another peak. A change in height of a single peak should not be rated as indicative of a mutation.

Note: It is recommended to use the RAS Extension Plug-in Report for result interpretation. For closer examination of samples with a reported potential low level mutation, we recommend to additionally analyze the sample manually in the application software (e.g., for comparison to the mutational frequency of the control sample).

Note: A treatment decision for cancer patients must not be based solely on KRAS and NRAS mutation status.

Table 9. LOB and LOD determined for specific mutations

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V70)
KRAS codon 59 (GCA)				
175G>A	A59T	0.5	3.5	546
176C>G	A59G	0.5	3.5	28518
KRAS codon 61 (CAA)				
183A>C	Q61H	0.8	2.8	554
182A>T	Q61L	1.2	3.1	553
182A>G	Q61R	1.6	3.5	552
183A>T	Q61H	0.7	2.6	555
181C>G	Q61E	1.2	3.1	550
KRAS codon 117 (AAA)				
351A>C	K117N	1.0	4.0	19940
351A>T	K117N	3.6	7.1	28519
KRAS codon 146 (GCA)				
436G>A	A146T	2.7	6.6	19404
436G>C	A146P	1.8	4.8	19905
437C>T	A146V	2.1	5.1	19900
NRAS codon 12 (GGT)				
34G>A	G12S	1.4	3.4	563
34G>T	G12C	0.6	2.5	562
34G>C	G12R	0.4	2.4	561
35G>A	G12D	1.8	3.8	564
35G>T	G12V	3.8	8.8	566
35G>C	G12A	0.5	2.5	565
NRAS codon 13 (GGT)				
37G>A	G13S	1.2	3.2	571
37G>T	G13C	1.2	3.2 (4)†	570
37G>C	G13R	0.3	2.3	569
38G>A	G13D	0.8	2.8	573
38G>T	G13V	0.0	2 (5)†	574
38G>C	G13A	0.8	2.8	575

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V70)
NRAS codon 59 (GCT)				
175G>A	A59T	3.8	6.9	578
176C>G	A59G	0.0	3.0	–
NRAS codon 61 (CAA)				
181C>A	Q61K	4.1	6.7	580
182A>G	Q61R	0.8	2.2	584
182A>T	Q61L	0.7	2.1	583
183A>T	Q61H	0.4	1.8	585
183A>C	Q61H	5.4	8.0	586
183A>G	Q61Q	2.1	5.8	587
NRAS codon 117 (AAG)				
351G>C	K117N	1.4	4.4	–
351G>T	K117N	3.0	6.0	–
NRAS codon 146 (GCC)				
436G>A	A146T	1.4	4.4	27174
436G>C	A146P	3.5	7.2	–
437C>T	A146V	4.8	7.8	–

* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at **www.sanger.ac.uk/genetics/CGP/cosmic**.

† Lowest mutation level in a sample resulting in a measured frequency ≥LOD.

Representative results

Representative Pyrogram results are shown in Figure 8 through Figure 15.

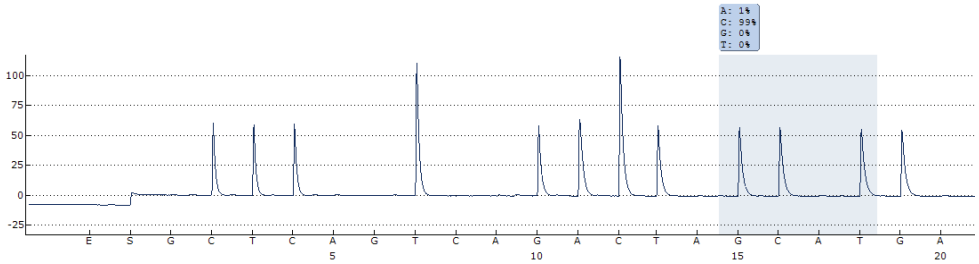


Figure 8. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the KRAS 59/61 assay.

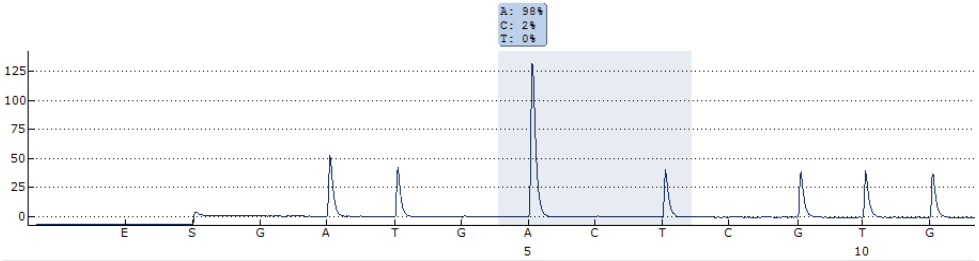


Figure 9. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the KRAS 117 assay.

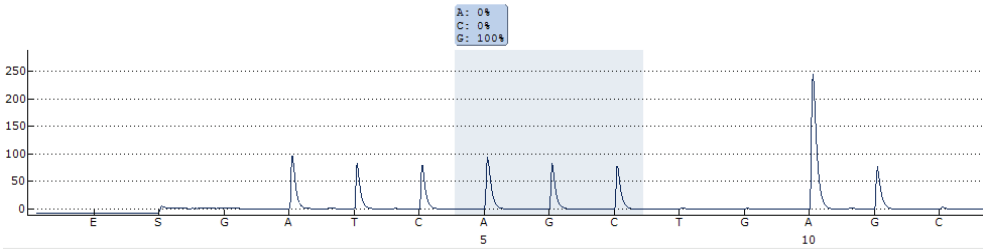


Figure 10. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the KRAS 146 assay.

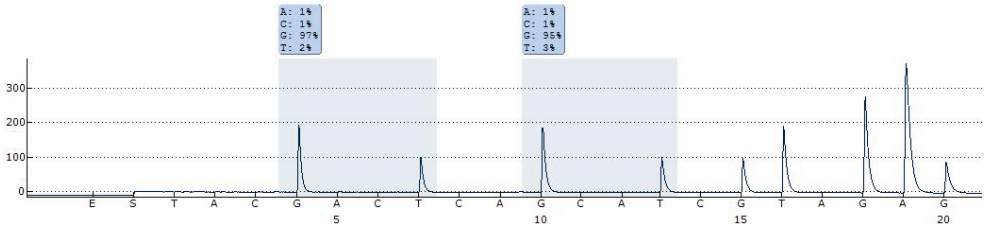


Figure 11. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 12/13 assay.

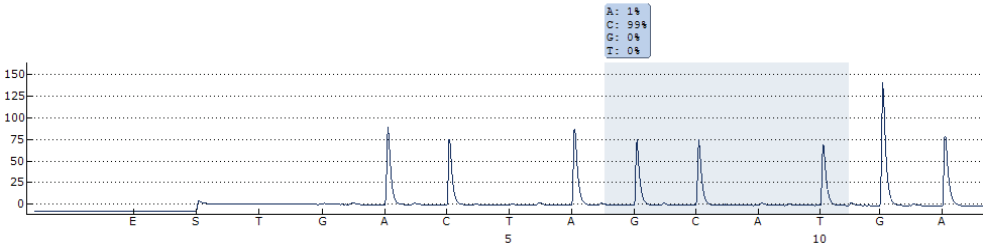


Figure 12. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 59 assay.

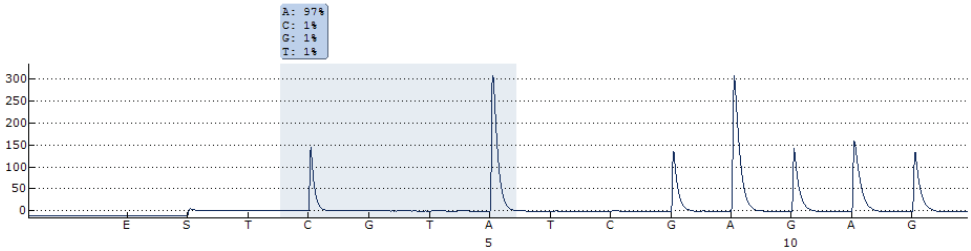


Figure 13. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 61 assay.

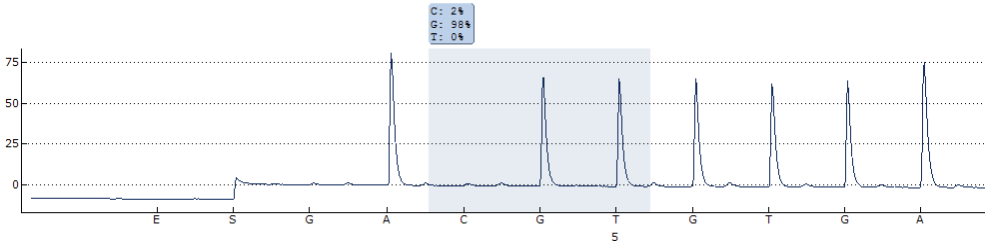


Figure 14. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 117 assay.

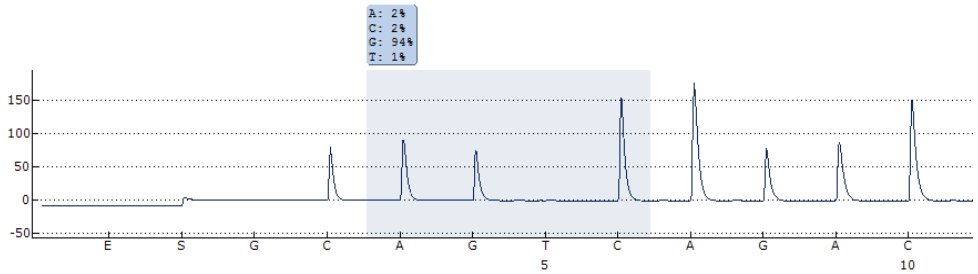


Figure 15. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 146 assay.

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
"Check" or "failed" result	
a) Low peak height	Handling errors in PCR setup or sample preparation prior to Pyrosequencing can result in low peaks. It is important that the samples are completely taken up by the vacuum tool. Take care that the vacuum tool is lowered slowly into the samples and that the geometry of the PCR plate or strips used for immobilization allows complete take up of the samples. Perform the function test for filter probes as described in the *PyroMark Q24 User Manual* on a regular basis, and exchange filter probes when indicated. In case of a "Check" warning, carefully compare the Pyrogram to the histogram, which can be displayed by right-clicking in the Pyrogram window. If the measured peaks match the height of the histogram bars, the result is valid. Otherwise, we recommend rerunning the sample.
b) Mutation not defined in "Sequence to Analyze"	Adjust the "Sequence to Analyze" in the assay setup (see "Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays", page 61), and reanalyze the run. Mutations that are not covered with "Sequences to Analyze" can be identified with the pattern simulation tool.
c) Unexpected rare mutation	A "Check" or "Failed" quality assessment can be caused by an unexpected pattern of peaks. This might indicate an unexpected mutation, which is not analyzed by the provided "Sequence to Analyze". These samples should be analyzed using the alternative "Sequence to Analyze" considering unexpected mutations. Mutations that are not covered with "Sequences to Analyze" can be identified with the pattern simulation tool.
d) High peak height deviation warning for a dispensation	The Pyrogram should be carefully compared to the histogram, which can be displayed by right-clicking in the Pyrogram window. In case the measured peaks do not match the height of the histogram bars and cannot be explained by rare mutations, we recommend rerunning the sample.

		Comments and suggestions
High background		
a)	Incorrect storage of nucleotides	Store nucleotides at 2–8°C. Storage at –15 to –25°C can cause an increase in the background.
b)	Short cooling time of samples prior to Pyrosequencing analysis	Keep the samples on a PyroMark Q24 plate holder at room temperature for 10–15 minutes. Do not shorten the cooling time.
c)	Contamination of cartridge	Carefully clean the cartridge as described in the product sheet. Store the cartridge protected from light and dust.
No signals in positive control (unmethylated control DNA)		
a)	Insufficient enzyme or substrate mix for all wells	Make sure to fill the PyroMark Q24 Cartridge according to the "Pre Run Information" in the "Tools" menu.
b)	Reagents incorrectly stored or diluted	Prepare the reagents according to the instructions in "Reagent Storage and Handling" on page 14 and "Protocol 5: Running the PyroMark Q24" on page 30.
c)	PCR or sample preparation failure	Carefully clean the cartridge as described in the product sheet. Store the cartridge protected from light and dust.

Quality Control

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

Limitations

The test is designed to detect 37 mutations in the KRAS or NRAS genes. Samples with results reported as "No Mutation Detected" may harbor KRAS or NRAS mutations not detected by the assay.

Detection of mutations is dependent on sample integrity and the amount of amplifiable DNA present in the specimen.

The *therascreen* RAS Extension Pyro Kit is used in a procedure employing a polymerase chain reaction (PCR). 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.

Any diagnostic results that are generated must be interpreted in conjunction with other clinical or laboratory findings.

It is the user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by the QIAGEN performance studies.

Performance Characteristics

Limit of blank and limit of detection

The limit of blank (LOB) and limit of detection (LOD) have been determined for a number of mutations using mixtures of plasmids (Table 10). LOB and LOD were determined according to the recommendations in the Clinical and Laboratory Standards Institute (CLSI) Guideline EP17 A2 "Protocol for determination of limits of detection and limits of quantitation; approved guideline". α- and β-errors (false positive and false negative, respectively) were set to 5%. LOB values represent the measured frequency obtained with a wild-type sample. LOD values represent the lowest signal (measured frequency) that can be regarded as positive for the respective mutation.

Table 10. LOB and LOD determined for specific mutations

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V70)
KRAS codon 59 (GCA)				
175G>A	A59T	0.5	3.5	546
176C>G	A59G	0.5	3.5	28518
KRAS codon 61 (CAA)				
183A>C	Q61H	0.8	2.8	554
182A>T	Q61L	1.2	3.1	553
182A>G	Q61R	1.6	3.5	552
183A>T	Q61H	0.7	2.6	555
181C>G	Q61E	1.2	3.1	550
KRAS codon 117 (AAA)				
351A>C	K117N	1.0	4.0	19940
351A>T	K117N	3.6	7.1	28519
KRAS codon 146 (GCA)				
436G>A	A146T	2.7	6.6	19404
436G>C	A146P	1.8	4.8	19905
437C>T	A146V	2.1	5.1	19900
NRAS codon 12 (GGT)				
34G>A	G12S	1.4	3.4	563
34G>T	G12C	0.6	2.5	562
34G>C	G12R	0.4	2.4	561
35G>A	G12D	1.8	3.8	564
35G>T	G12V	3.8	8.8	566
35G>C	G12A	0.5	2.5	565
NRAS codon 13 (GGT)				
37G>A	G13S	1.2	3.2	571
37G>T	G13C	1.2	3.2 (4)†	570
37G>C	G13R	0.3	2.3	569
38G>A	G13D	0.8	2.8	573
38G>T	G13V	0.0	2 (5)†	574
38G>C	G13A	0.8	2.8	575

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V70)
NRAS codon 59 (GCT)				
175G>A	A59T	3.8	6.9	578
176C>G	A59G	0.0	3.0	–
NRAS codon 61 (CAA)				
181C>A	Q61K	4.1	6.7	580
182A>G	Q61R	0.8	2.2	584
182A>T	Q61L	0.7	2.1	583
183A>T	Q61H	0.4	1.8	585
183A>C	Q61H	5.4	8.0	586
183A>G	Q61Q	2.1	5.8	587
NRAS codon 117 (AAG)				
351G>C	K117N	1.4	4.4	–
351G>T	K117N	3.0	6.0	–
NRAS codon 146 (GCC)				
436G>A	A146T	1.4	4.4	27174
436G>C	A146P	3.5	7.2	–
437C>T	A146V	4.8	7.8	–

* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at **www.sanger.ac.uk/genetics/CGP/cosmic**.

† Lowest mutation level in a sample resulting in a measured frequency ≥LOD.

Mutations GGT >TGT and GGT > GTT in NRAS codon 13

For these mutations, blank measurements were mostly 0 percentage units resulting in a non-Gaussian distribution. LOD was therefore determined using a different method, according to the recommendations in the CLSI Guideline EP17-A. The lowest signal that indicates the presence of a mutation (LOD) in these positions was set to 2 percentage units above the respective baseline level as defined by the 95th percentile of blank measurements. When analyzing a sample with the mutation level given in brackets in Table 9, 95% of results (n=72) gave a signal that can be regarded as positive (≥LOD). For LOB/LOD see Table 10.

Note: PCR and Pyrosequencing primers for NRAS codons 12, 13 and 61 are taken without changes from the *therascreen* NRAS Pyro Kit (cat. no. 971530). Performance data for these NRAS codon remain unchanged.

Linearity

Linearity was determined using plasmid mixtures carrying the wild-type, or mutant sequence of the mutations 176C>G in KRAS codon 59, 351A>T in KRAS codon 117, 436G>C in KRAS codon 146, 34G>A in NRAS codon 12, 37G>A in NRAS codon 13, 175G>A in NRAS codon 59, 182A>G in NRAS codon 61, 351G>C in NRAS codon 117, and 437C>T in NRAS codon 146. The plasmids were mixed in proportion to yield four levels of mutations (5, 10, 30 and 50%). Each mixture was analyzed with three different lots of the *therascreen* RAS Extension Pyro Kit in 3 Pyrosequencing runs with three replicates each.

The results (n = 9 for each mutation level) were analyzed according to the CLSI Guideline EP6-A2 "Evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline" using the Analyse-it® Software v2.21. These results are shown in Figure 16.

The results were linear within an allowable nonlinearity of 5% units in the tested range of 5–50% mutation levels. Similar results were obtained for all covered mutations in KRAS codons 59, 117, 146, and NRAS codons 12, 13, 59, 61, 117 and 146.

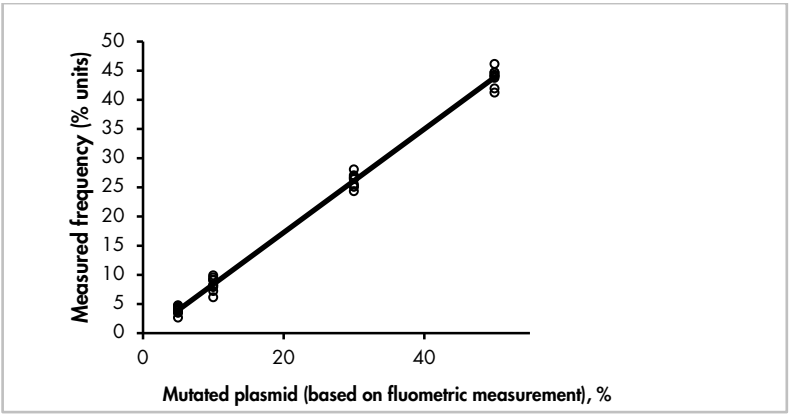


Figure 16. Linearity of the mutation 176C>G in KRAS codon 59.

Similar results were obtained for all covered mutations in KRAS codons 59, 117, 146, and NRAS 12, 13, 59, 61, 117 and 146.

Precision

The precision data allows for the determination of the total variability of the assays, and was obtained at three different levels by analysis of the above mentioned plasmid mixtures, with three replicates each.

Repeatability (intra-assay and inter-batch variability) was calculated based on the data for determination of linearity (three runs on the same day using varying lots of the *therascreen* RAS Extension Pyro Kit). Intermediate precision (intra-laboratory variability) was determined over three runs within one laboratory on three separate days. The runs were performed by different operators, using PyroMark Q24 instruments, as well as and many *therascreen* RAS Extension Pyro Kits. Reproducibility (inter-laboratory variability) was calculated from two runs each in two independent laboratories, and using varying *therascreen* RAS Extension Pyro Kit lots.

Precision estimates are expressed as a standard deviation of the measured mutation frequencies in % units (Table 11).

Table 11. Precision of mutations*

	Repeatability		Intermediate precision		Reproducibility	
% Mutated plasmid†	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
176C>G in KRAS codon 59						
5	4.0	0.7	3.8	0.6	4.2	1.1
10	8.4	1.2	8.5	1.0	8.4	1.4
30	26.1	1.2	26.3	1.1	26.8	1.2
50	43.9	1.5	44.0	0.7	43.7	1.3
351A>T in KRAS codon 117						
5	5.5	1.6	5.5	2.2	7.1	2.0
10	11.0	1.7	10.8	1.4	12.5	2.9
30	30.6	1.7	30.6	2.0	31.9	2.7
50	52.8	2.0	53.5	1.3	54.5	1.6
436G>C in KRAS codon 146						
5	4.2	0.6	4.1	0.5	3.7	1.2
10	9.6	0.9	9.1	0.9	8.6	1.3
30	29.0	0.9	28.8	1.0	28.1	1.1
50	47.5	1.5	46.8	0.7	45.6	1.9
34G>A in NRAS codon 12†						
5	7.5	1.2	7.3	1.0	6.7	1.3
10	14.6	1.3	13.5	1.1	13.7	1.3
30	37.8	1.9	37.9	1.5	36.1	2.9
50	59.8	1.7	60.4	2.0	57.5	3.1
175G>A in NRAS codon 59						
5	7.8	0.9	7.3	0.5	7.1	1.3
10	11.9	1.0	11.6	2.0	12.5	1.7
30	29.5	1.1	29.6	1.2	29.9	1.9
50	49.0	1.1	48.3	1.3	48.9	1.4

	Repeatability		Intermediate precision		Reproducibility	
% Mutated plasmid†	Mean	SD	Mean	SD	Mean	SD
182A>G in NRAS codon 61						
5	6.4	0.9	6.8	0.7	7.2	1.0
10	11.7	0.9	11.8	1.1	11.8	1.0
30	34.1	1.3	34.6	1.7	33.8	2.5
50	53.1	1.5	53.3	1.8	53.1	2.0
351G>C in NRAS codon 117						
5	4.9	0.2	5.0	0.3	4.5	0.8
10	9.4	0.4	10.3	1.5	9.4	0.5
30	28.7	0.9	28.8	0.7	28.3	1.3
50	48.5	0.4	48.8	0.6	48.8	0.6
437C>T in NRAS codon 146						
5	4.4	0.7	4.6	0.5	4.1	0.9
10	8.8	0.9	8.7	0.8	9.1	0.8
30	28.4	1.1	27.9	0.6	28.4	0.8
50	47.9	1.1	48.1	1.4	48.0	1.1

* All values are given as % units. SD: standard deviation (n = 9 for repeatability and intermediate precision, n = 12 for reproducibility).

† Based on fluometric measurement, for 34G>A in NRAS codon 12 based on OD_{260}.

Diagnostic evaluation

The *therascreen* RAS Extension Pyro Kit was evaluated in comparison with Sanger sequencing in two different studies.

A first study was previously performed to evaluate the *therascreen* NRAS Pyro kit in comparison with Sanger sequencing. DNA was extracted from 100 formalin-fixed paraffin-embedded (FFPE) tumor samples from bone marrow and analyzed for mutations in codons 12/13, and codon 61.

As assays covering NRAS codon 12/13 and 61 in *therascreen* NRAS Pyro kit are included in *therascreen* RAS Extension Pyro Kit without changes, results are shown from the *therascreen* NRAS Pyro Kit evaluation.

In the second study, DNA was extracted from 110 formalin-fixed paraffin-embedded (FFPE) mCRC tumor samples, and analyzed for mutations in codons 59, 61, 117 and 146 of the human KRAS and codons 59, 117 and 146 for the human NRAS genes. Low frequency mutations were analyzed using plasmid DNA that was spiked with wild-type FFPE DNA.

In both studies, DNA was isolated using the QIAamp DNA FFPE Tissue Kit, and then analyzed using assays included in the *therascreen* RAS Extension Pyro Kit on the PyroMark Q24. The Applied Biosystems® 3730xl Genetic Analyzer performed Sanger sequencing.

Evaluation of NRAS codon 12, 13 and 61

Of 100 samples analyzed by Sanger sequencing, the mutational status was determined in 97 samples for both codon 12/13, and codon 61. In four of the 100 samples, a mutation in codon 12 or codon 13 was detected by Sanger sequencing.

In two of the 100 samples, the mutational status was reproduced using the *therascreen* NRAS Pyro Kit, and no mutation was reported. The results are illustrated in Table 12. No mutations were detected in codon 61.

Excluding samples that failed using one or both methods, the *therascreen* NRAS Pyro Kit and Sanger sequencing showed 98% and 100% concordance in the results for codons 12/13 and codon 61, respectively; see Table 12).

Table 12. Results of the analyzed samples for NRAS 12, 13, and 61

		Sanger sequencing				
		Mutant in codon 12/13	**Mutant in codon 61**	**Wild-type**	**Unknown**	**Total**
***therascreen* NRAS Pyro Kit**	**Mutant in codon 12/13**	2	–	–	–	**2**
	Mutant in codon 61	–	–	–	–	**–**
	Wild-type	2	–	90	3	**95**
	Unknown	–	–	3	–	**3**
	Total	**4**	**–**	**93**	**3**	**100**

Evaluation of KRAS codon 59, 61, 117, 146, and NRAS codon 59, 117, 146

DNA was extracted from 110 formalin-fixed paraffin-embedded (FFPE) mCRC tumor samples, and analyzed for mutations in codons 59, 61, 117 and 146 of the human KRAS gene and 59, 117 and 146 of the human NRAS gene. Due to the expected low abundance in clinical samples, all mutations covered by the *therascreen* RAS Extension Kit were analyzed in 56 additional samples using plasmid DNA that was spiked into wild-type FFPE DNA. All mutations were found by both, Pyro- and Sanger-Sequencing.

Out of the 166 sample analyzed, overall concordant results between *therascreen* RAS Extension Pyro Kit and Sanger Sequencing were found for 137 samples (83%).

Discordant cases could be explained due to several factors.

Due to high background, 20 samples failed the NRAS 59 Sanger Sequencing analysis.

Sanger sequencing did not detect mutations in KRAS 59 and KRAS 61 in 1 and 3 samples, respectively. All four mutations had low frequency results from Pyrosequencing (7.5–13.1%). This can be explained with the lower sensitivity of Sanger Sequencing (15–20%) compared to Pyrosequencing (5%) (2). All other valid samples were wild-type for both techniques

One sample has been rated as unknown for Pyrosequencing due to a double mutation detected (KRAS 59 – 61).

Four samples containing spiked plasmid DNA showed an additional A>G mutation at KRAS coding sequence position 350, which is not covered by the *therascreen* RAS Extension Pyro Kit. Mutations were detected by manual analysis.

Table 13. Results of the analyzed samples for KRAS codon 59, 61, 117, 146, and NRAS codon 59, 117, 146

		KRAS 59	KRAS 61	KRAS 117	KRAS 146	KRAS[a]	NRAS[b]	wt	UK	Total
therascreen RAS Extension Pyro Kit	KRAS 59	8	–	–	–	–	–	–	1	**9**
	KRAS 61	–	6	–	–	–	–	2	1	**9**
	KRAS 117	–	–	4	–	–	–	–	–	**4**
	KRAS 146	–	–	–	3	4	–	–	–	**7**
	KRAS[a]	–	–	–	–	16	–	–	–	**16**
	NRAS[b]	–	–	–	–	–	28	–	–	**28**
	wt	–	–	–	–	–	–	71	16	**87**
	UK	1	–	–	–	–	–	3	2	**6**
	Total	**9**	**6**	**4**	**3**	**20**	**28**	**76**	**20**	**166**

UK: Unknown; wt: Wild-type.

[a] KRAS spiked samples carrying both KRAS 117 and 146 mutations.

[b] NRAS spiked samples carrying mutation for NRAS 59, 117 and 146.

* One sample was detected mutant for KRAS 146 but showed an invalid result for NRAS 117.

Sensitivity and specificity of the assays per codon are reported in the Table 14.

Table 14. Sensitivity and specificity of KRAS codon 59, 61, 117, 146, and NRAS codon 59, 117, 146 assays

	Sensitivity	Specificity	Mutation covered
Mutation KRAS 59	100%	99%	175G>A / 176C>G
Mutation KRAS 61	100%	97%	181C>G / 182A>T / 183A>C / 183A>T
Mutation KRAS 117	100%	100%	351A>C / 351A>T
Mutation KRAS 146	100%	100%	436G>A / 436G>C / 437C>T
Mutation NRAS 59	100%	100%	175G>A / 176C>G
Mutation NRAS 117	100%	100%	351G>C / 351G>T
Mutation NRAS 146	100%	100%	436G>A / 436G>C / 437C>T

Note: In all runs used for determination of performance characteristics, the signal was over 30 RLU, as is routinely obtained from 10 ng of DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue. The Pyrosequencing data was analyzed using the RAS Extension Plug-in Report for KRAS codons 59, 117 146 and NRAS codons 59, 117 and 146.

References

1. Douillard, J.Y., Oliner, K.S., Siena, S., Tabernero, J., Burkes, R., Barugel, M., et al. (2013) Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N. Engl. J. Med. **369**, 1023.

2. Tsiatis, A.C., Norris-Kirby, A., Rich, R.G., Hafez, M.J., Gocke, C.D., Eshleman, J.R., et al. (2010) Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of *KRAS* mutations: diagnostic and clinical implications. J. Mol. Diagn. **12**, 425.

Symbols

The following symbols may appear on the packaging and labeling:

Symbol	Symbol definition
 <N>	Contains reagents sufficient for <N> reactions
	Use by
	In vitro diagnostic medical device
	
	Catalog number
	Lot number
	Material number
	Components
	Contains
	Number
	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Consult instructions for use

Symbol	Symbol definition
	Caution

Contact Information

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

Appendix A: Setting Up *therascreen* RAS Extension Pyro Assays

If the RAS Extension Plug-in Report has been installed, predefined Assay Setups for KRAS codons 59/61, 117, and 146 and NRAS codons 12/13, 59, 61, 117 and 146 are available in the shortcut browser of the PyroMark Q24 software. Follow the path "Example Files/PyroMark Setups/RAS Extension". In this case, the following steps do not need to be performed.

The RAS Extension Plug-in Report can be downloaded from the relevant catalog page on **www.qiagen.com** under the "Product Resources" tab in the Protocol Files section.

We strongly recommend the use of the RAS Extension Plug-in Report over manual analysis.

After installation of the Plug-in or each time new software is installed or upgraded on the computer, the correct function of the plug-in should be verified as described in the RAS Extension Plug-In Quick Guide.

If the RAS Extension Plug-in Report has not been installed, the assay file must be set up manually before running the *therascreen* RAS Extension Pyro assay for the first time. Set up the assay for KRAS codons 59/61, 117, and 146 and NRAS codons 12, 13, 59, 61, 117 and 146 by using the PyroMark Q24 Software, as described below.

Procedure

1. Click  in the toolbar and select "New AQ Assay".
2. Table 15 shows the "Sequences to Analyze" to analyze for all eight RAS Extension Pyro assays. Type the assay specific sequence in "Sequence to Analyze" field.
3. The "Sequence to Analyze" can also be changed after the run to analyze for mutations at different positions (see "Protocol 6: Analysis of a PyroMark Q24 run", page 33).

4. To check if mutations are present in other nucleotides, change the "Sequence to Analyze" according to Table 15. Changing the "Sequence to Analyze" is possible after the run (if not locked).

   Note: Ensure that the threshold for single peak height is set to 30 RLU. Additionally, ensure that the A-peak reduction factor is set to 0.86 for analysis of NRAS codon 61.

5. Manually enter the assay specific "Dispensation Order" from Table 15.

   Note: Do not use the "Generate Dispensation Order" button. Both "Sequence to Analyze" and "Dispensation Order" must be typed in manually.

6. Click the "Analysis Parameters" tab, and increase "Peak Height Threshold - Required peak height for Passed quality:" to 30.

7. Click  in the toolbar, and save the assay as "*KRAS 59/61*" or "*KRAS 117*" or "*KRAS 146*" or "*NRAS 12/13*" or "*NRAS 59*" or "*NRAS 61*" or "*NRAS 117*" or "*NRAS 146*".

Table 15. Assay setup: "Sequence to analyze" and "Dispensation order" for the eight assays of the *therascreen* RAS extension Pyro Kit

***therascreen* RAS Extension assay**	**Sequence to analyze**	**Dispensation order**
KRAS 59/61	CTCDTGACCTGCTGT	GCTCAGTCAGACTAGCATGA
KRAS 117	ATAAHTGTGA	GATGACTCGTG
KRAS 146	ATCAVCAAAGA	GATCAGCTGAGC
NRAS 12/13	GNTGNTGTTGGGAAAAGC	TACGACTCAGCATCGTAGAG
NRAS 59	ACAGNTGGAC	TGACTAGCATGA
NRAS 61	CNAGAAGAGTA	TCGTATCGAGAG
NRAS 117	ABTGTGATTT	GACGTGTGA
NRAS 146	CANCCAAGACCA	GCAGTCAGAC

Table 16. Common mutations in the human KRAS gene detected by the *therascreen* RAS Extension Pyro Kit with respective "Sequence to analyze"

Nucleic acid substitution	Amino acid substitution	Sequence to analyze	Cosmic ID (V70)*
KRAS codon 59 (GCA)			
175G>A	A59T	CTCTTGACCTG**N**TGT	546
176C>G	A59G	CTCTTGACCT**N**CTGT	28518
KRAS codon 61 (CAA)			
183A>C	Q61H	CTC**D**TGACCTGCTGT	554
182A>T	Q61L	CTCT**H**GACCTGCTGT	553
182A>G	Q61R	CTCT**H**GACCTGCTGT	552
183A>T	Q61H	CTC**D**TGACCTGCTGT	555
181C>G	Q61E	CTCTT**S**ACCTGCTGT	550
KRAS codon 117 (AAA)			
351A>C	K117N	ATAA**H**TGTGA	19940
351A>T	K117N	ATAAHTGTGA	28519
KRAS codon 146 (GCA)			
436G>A	A146T	ATCA**V**CAAAGA	19404
436G>C	A146P	ATCA**V**CAAAGA	19905
437C>T	A146V	ATCAG**B**AAAGA	19900

* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at **www.sanger.ac.uk/genetics/CGP/cosmic/**.

Table 17. Common mutations in the human NRAS gene detected by the *therascreen* RAS Extension Pyro Kit with respective "Sequence to analyze"

Nucleic acid substitution	Amino acid substitution	Sequence to analyze	Cosmic ID (V70)*
NRAS codon 12 (GGT)			
34G>A	G12S	**N**GT**N**GTGTTGGGAAAAGC	563
34G>T	G12C	**N**GT**N**GTGTTGGGAAAAGC	562
34G>C	G12R	**N**GT**N**GTGTTGGGAAAAGC	561
35G>A	G12D	G**N**TG**N**TGTTGGGAAAAGC	564
35G>T	G12V	G**N**TG**N**TGTTGGGAAAAGC	566
35G>C	G12A	G**N**TG**N**TGTTGGGAAAAGC	565
NRAS codon 13 (GGT)			
37G>A	G13S	**N**GT**N**GTGTTGGGAAAAGC	571
37G>T	G13C	**N**GT**N**GTGTTGGGAAAAGC	570
37G>C	G13R	**N**GT**N**GTGTTGGGAAAAGC	569
38G>A	G13D	G**N**TG**N**TGTTGGGAAAAGC	573
38G>T	G13V	G**N**TG**N**TGTTGGGAAAAGC	574
38G>C	G13A	G**N**TG**N**TGTTGGGAAAAGC	575
NRAS codon 59 (GCT)			
175G>A	A59T	ACA**V**CTGGAC	578
176C>G	A59G	ACAG**N**TGGAC	–
NRAS codon 117 (AAG)			
351G>C	K117N	A**B**TGTGATTT	–
351G>T	K117N	A**B**TGTGATTT	–
NRAS codon 61 (CAA)			
181C>A	Q61K	**V**AAGAAGAGTA	580
182A>G	Q61R	C**N**AGAAGAGTA	584
182A>T	Q61L	C**N**AGAAGAGTA	583
183A>T	Q61H	CA**N**GAAGAGTA	585
183A>C	Q61H	CA**N**GAAGAGTA	586

Nucleic acid substitution	Amino acid substitution	Sequence to analyze	Cosmic ID (V70)*
183A>G	Q61Q	CA**N**GAAGAGTA	587
NRAS codon 146 (GCC)			
436G>A	A146T	CA**N**CCAAGACCA	27174
436G>C	A146P	CA**N**CCAAGACCA	–
437C>T	A146V	CAG**B**CAAGACCA	–

* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at **www.sanger.ac.uk/genetics/CGP/cosmic/**.

Appendix B: Emptying the Waste Container and Troughs

 WARNING	Hazardous chemicals The Denaturation Solution used with the vacuum workstation contains sodium hydroxide, which is irritating to eyes and skin. Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Safety Data Sheets (SDSs) or OSHA,* ACGIH,† or COSHH‡ documents. Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.

* OSHA: Occupational Safety and Health Administration (United States of America).

† ACGIH: American Conference of Government Industrial Hygienists (United States of America).

‡ COSHH: Control of Substances Hazardous to Health (United Kingdom).

Be sure to observe federal, state, and local environmental regulations for the disposal of laboratory waste.

Important point before starting

- This protocol requires high-purity water.

Procedure

1. Ensure that no vacuum is applied to the vacuum tool. Make sure that the vacuum is closed (Off) and the vacuum pump is turned off.
2. Discard any solutions left in the troughs.
3. Rinse the troughs with high-purity water, or replace them if necessary.
4. Empty the waste container.
5. The cap can be removed without disconnecting the tubing.

If the vacuum workstation must be cleaned (for example, due to dust or spillage), follow the instructions in the *PyroMark Q24 User Manual*.

Ordering Information

Product	Contents	Cat. no.
therascreen RAS Extension Pyro Kit (24)	For 24 reactions: Sequencing Primers, PCR Primers, Unmethylated Control DNA, PyroMark PCR Master Mix, CoralLoad Concentrate, Buffers, and Reagents	971590
PyroMark Q24 MDx	Sequence-based detection platform for Pyrosequencing of 24 samples in parallel	9001513
PyroMark Q24 MDx Vacuum Workstation	Vacuum Workstation for preparing 24 samples in parallel, from PCR product to single-stranded template	9001515
PyroMark Q24 MDx Software	Analysis software	9019063
Accessories		
PyroMark Q24 Plate (100)	24-well sequencing reaction plate	979301
PyroMark Q24 Cartridge (3)	Cartridges for dispensing nucleotides and reagents	979302
PyroMark Vacuum Prep Filter Probe (100)	Reusable filter probes for PyroMark Vacuum Workstation Q96 and Q24	979010
PyroMark Control Oligo	For installation check of system	979303
PyroMark Q24 Validation Oligo	For performance confirmation of system	979304

Product	Contents	Cat. no.
Related products		
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404

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

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