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May 2016

# RAS Extension Pyro<sup>®</sup> V2 Kit Handbook

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



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## Intended Use

The RAS Extension Pyro V2 Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

## Summary and Explanation

The RAS Extension Pyro V2 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 (see Figure 1).

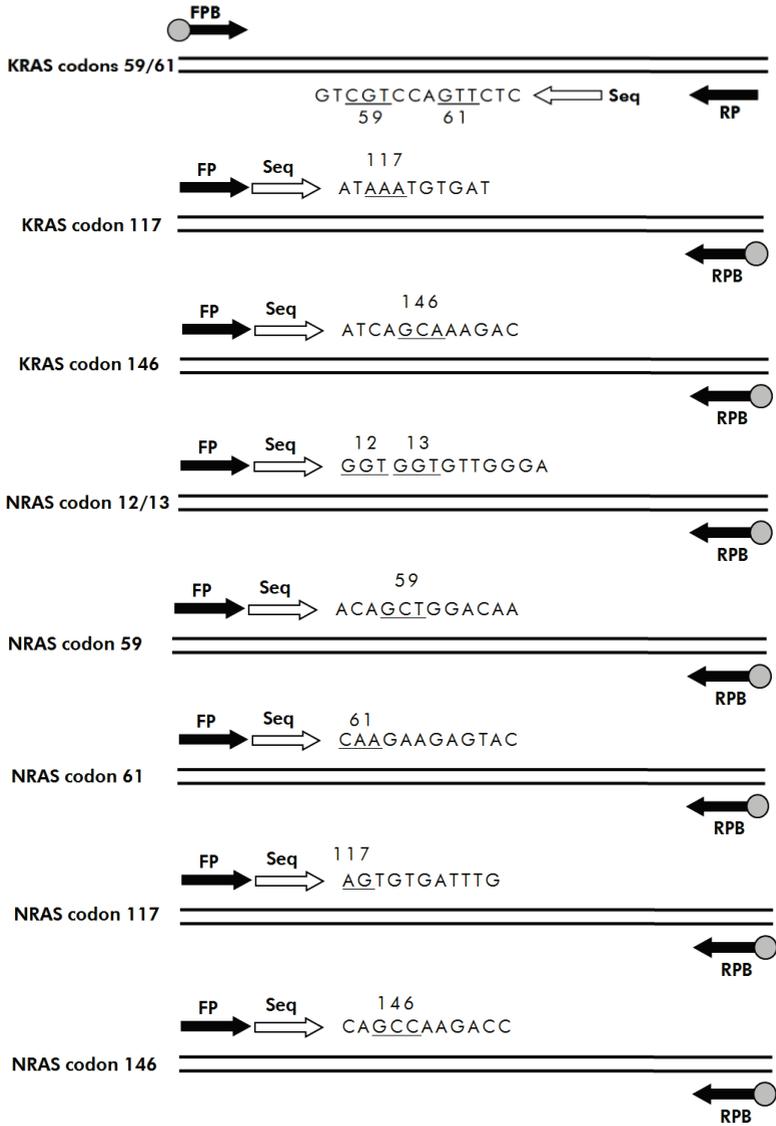


Figure 1. Assays of the RAS Extension Pyro V2 Kit.

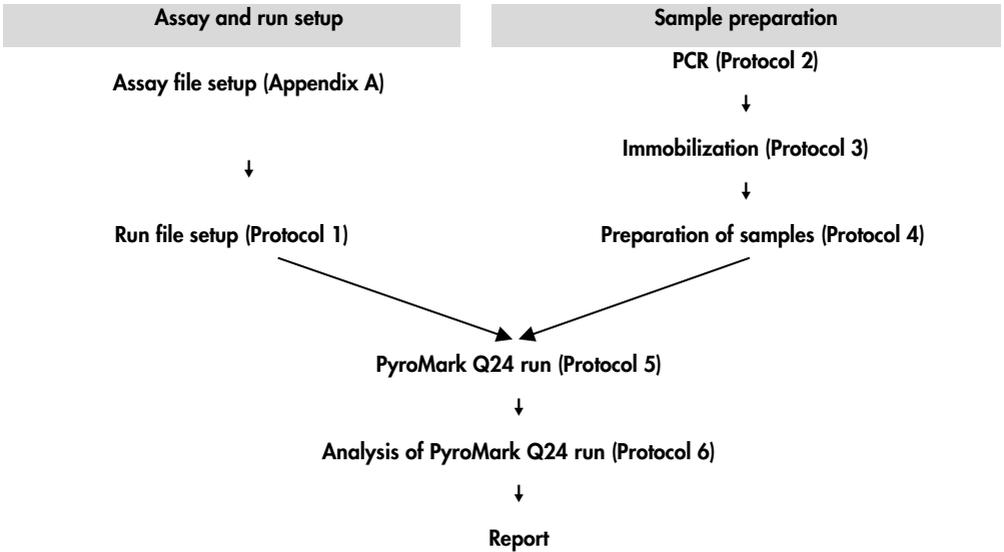
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The eight regions are amplified separately by PCR, and sequenced through the defined region. Mutations in the covered region will lead to distinct patterns in the Pyrogram® trace that are distinguishable from traces obtained from wild-type samples. Mutations that can be analyzed using the PyroMark® Q24 software are listed in Table 10 (Appendix A: Setting Up RAS Extension Pyro V2 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 34, and “Appendix A: Setting Up RAS Extension Pyro V2 Assays”, page 48).



**Figure 2.** Workflow of RAS Extension Pyro V2 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 34).

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

<b>RAS Extension Pyro V2 Kit</b>	<b>(24)</b>
<b>Catalog no.</b>	<b>970590</b>
<b>Number of preps</b>	<b>24</b>
Seq Primer KRAS 59/61	24 $\mu$ l
Seq Primer KRAS 117	24 $\mu$ l
Seq Primer KRAS 146	24 $\mu$ l
Seq Primer NRAS 12/13	24 $\mu$ l
Seq Primer NRAS 59	24 $\mu$ l
Seq Primer NRAS 61	24 $\mu$ l
Seq Primer NRAS 117	24 $\mu$ l
Seq Primer NRAS 146	24 $\mu$ l
PCR Primer KRAS 59/61	24 $\mu$ l
PCR Primer KRAS 117	24 $\mu$ l
PCR Primer KRAS 146	24 $\mu$ l
PCR Primer NRAS 12/13	24 $\mu$ l
PCR Primer NRAS 59	24 $\mu$ l
PCR Primer NRAS 61	24 $\mu$ l
PCR Primer NRAS 117	24 $\mu$ l
PCR Primer NRAS 146	24 $\mu$ l
PyroMark PCR Master Mix, 2x	4 x 850 $\mu$ l
CoralLoad® Concentrate, 10x	1.2 ml
H <sub>2</sub> O	6 x 1.9 ml
Unmethylated Control DNA, 10 ng/ $\mu$ l	3 x 100 $\mu$ l

## 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
dATP $\alpha$ S	2 x 1180 $\mu$ l
dCTP	2 x 1180 $\mu$ l
dGTP	2 x 1180 $\mu$ l
dTTP	2 x 1180 $\mu$ l
Handbook	1

\* 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](http://www.gelifesciences.com))
- High-purity water (Milli-Q® 18.2 M $\Omega$  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.

† 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 RAS Extension Pyro V2 Kit.

**Table 1. Plate mixers recommended for use with the RAS Extension Pyro V2 Kit**

<b>Manufacturer</b>	<b>Product</b>	<b>Catalog no.</b>
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 RAS Extension Pyro V2 Kit.

**Table 2. 24-well plates recommended for use with the RAS Extension Pyro V2 Kit**

<b>Manufacturer</b>	<b>Product</b>	<b>Catalog no.</b>
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

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# Warnings and Precautions

For molecular biology applications

## Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at [www.qiagen.com/safety](http://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.

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## Reagent Storage and Handling

The RAS Extension Pyro V2 Kit is shipped in two boxes. The RAS Extension Pyro V2 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  $-25^{\circ}\text{C}$  upon arrival.

The Pyro Buffers and Reagents (box 2/2) containing Buffers, Enzyme Mixture, Substrate Mixture, dATPaS, dCTP, dGTP, and dTTP (the reagents for Pyrosequencing analysis) is shipped on cool packs. These components should be stored at  $2-8^{\circ}\text{C}$  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  $2-8^{\circ}\text{C}$ . Reconstituted enzyme and substrate mixtures can be frozen, and stored in their vials at  $-15$  to  $-25^{\circ}\text{C}$ . 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 RAS Extension Pyro V2 Kit is stable until the kit expiration date.

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# 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 RAS Extension Pyro V2 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  $\mu$ m serial sections from the paraffin block and mount them on glass slides.
- A trained individual (e.g., a pathologist) must assess a Hematoxylin & Eosin (H&E)-stained section for tumor content and area determination. Mark the stained slide to distinguish tumor from normal tissue. Use serial sections for DNA extraction.
- Use sections with >20% tumor content by area for processing without macrodissection (see next point).

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- 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<sup>2</sup> in area, process two or more sections to increase the total tumor area to at least 4 mm<sup>2</sup> (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 kits shown in Table 3 are recommended for DNA purification for the indicated human sample type, as well as for use with the RAS Extension Pyro V2 Kit. To use these kits, follow the DNA purification instructions in the respective kit handbook.

**Table 3. DNA purification kit recommended for use with the RAS Extension Pyro V2 Kit**

Sample type	Nucleic acid isolation kit	Catalog number (QIAGEN)
Paraffin-embedded tissue	QIAamp® DNA FFPE Tissue Kit (50)	56404
	EZ1® DNA Tissue Kit (48) *	953034
	PAXgene® Tissue Containers (10)	765112
	PAXgene Tissue DNA Kit (50)	767134

\* Follow the protocol for use with paraffin-embedded tissue. The EZ1 DNA Tissue Kit is to be used in combination with the EZ1 Advanced (cat. no. 9001410 or 9001411) and the EZ1 Advanced DNA Paraffin Section Card (cat. no. 9018298), with the EZ1 Advanced XL (cat. no. 9001492) and the EZ1 Advanced XL DNA Paraffin Section Card (cat. no. 9018700), or with the BioRobot® EZ1 (cat. no. 9000705; no longer available) and the EZ1 DNA Paraffin Section Card (cat. no. 9015862).

## 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 RAS Extension Pyro V2 Assays” on page 48. 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 crosstalk 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 19).
3. Set up the plate by adding assays for all eight assays of the RAS Extension Pyro V2 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 24).

**Note:** To run the plate on the PyroMark Q24 system, see “Protocol 5: Running the PyroMark Q24”, page 31.

## 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 RAS Extension Pyro V2 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 RAS Extension Pyro V2 Kit can be traced.

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

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## 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.

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## Protocol 2: PCR using the PCR reagents supplied with the RAS Extension Pyro V2 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 RAS Extension Pyro V2 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 (see 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 <b>or</b> PCR Primer KRAS 117 <b>or</b> PCR Primer KRAS 146 <b>or</b> PCR Primer NRAS 12/13 <b>or</b> PCR Primer NRAS 59 <b>or</b> PCR Primer NRAS 61 <b>or</b> PCR Primer NRAS 117 <b>or</b> PCR Primer NRAS 146	1
Water (H <sub>2</sub> O, supplied)	4
<b>Total volume</b>	<b>20</b>

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 (see 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
<b>Total volume</b>	<b>25</b>

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

**Table 6. Optimized cycling protocol**

	<b>Time</b>	<b>Temperature</b>	<b>Comments</b>
<b>Initial activation step:</b>	15 min	95°C	HotStarTaq DNA polymerase is activated by this heating step
<b>3-step cycling:</b>			
Denaturation	20 s	95°C	
Annealing	30 s	53°C	
Extension	20 s	72°C	
Number of cycles	42	–	
<b>Final extension:</b>	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 24.  
The PCR samples can be stored at 2–8°C for up to 3 days.

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## 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 <sub>2</sub> O, supplied)	29
Streptavidin Sepharose High Performance	1
<b>Total volume</b>	<b>70</b>

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 18).  
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 RAS Extension Pyro V2 KitProtocol 2: PCR using the PCR reagents supplied with the RAS Extension Pyro V2 Kit”, page 21).  
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 26.

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## 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 18), 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**

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

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 18).

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.

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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 53.

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 31.

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## 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 18), 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

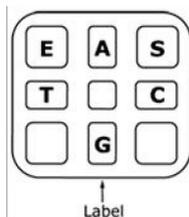
1. Dissolve the freeze-dried enzyme and substrate mixtures in 620 µl water (H<sub>2</sub>O, supplied).
2. Mix by swirling the vial gently.

**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 34.

---

## Protocol 6: Analysis of a PyroMark Q24 Run

This protocol describes the mutation analysis of a finished RAS Extension Pyro V2 run using PyroMark Q24 Software.

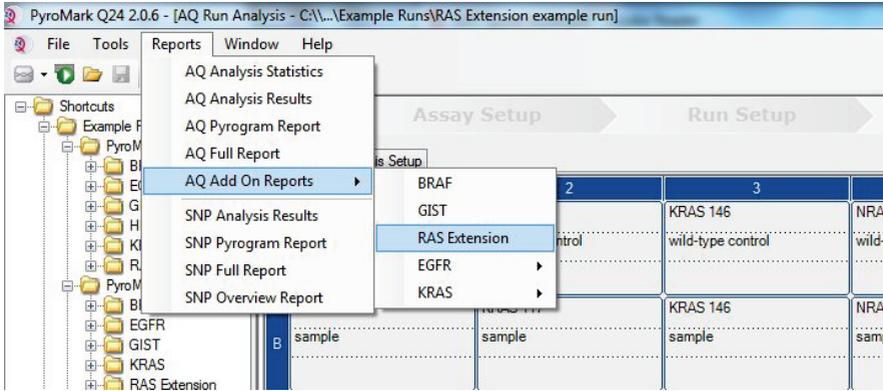
### Procedure

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. There are two methods for analyzing the run. If using the RAS Extension Plug-in Report, go to step 5. If using the AQ analysis integral to the PyroMark Q24, go to step 6.

**Note:** We strongly recommend using the RAS Extension Plug-in Report for result interpretation. The RAS Extension Plug-in Report can be downloaded from the relevant catalog page on [www.qiagen.com](http://www.qiagen.com) under the "Product Resources" tab in the Protocol Files section. This report ensures that the respective limit of detection (LOD) values and different "Sequences to Analyze" are used to automatically detect all mutations.

5. 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 61 must in addition, be analyzed with the separate KRAS plug-in by selecting "AQ Add On Reports/RAS Extension" 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, next page), 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				⚠

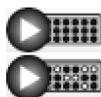
⚠ See detailed results below.

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.

### 6. 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.**

The standard “Sequence to Analyze” as defined in the Analysis Setup, addresses the most frequent point mutations in the RAS Extension Pyro V2 assays. If a sample contains a less frequent mutation, the sequence to analyze can be changed to also analyze the mutation status at this position, as described in “Appendix A: Setting Up RAS Extension Pyro V2 Assays”, page 48.

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/](http://www.sanger.ac.uk/genetics/CGP/cosmic/).

**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 RAS Extension Pyro V2 Assays”, page 48, 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.

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

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 11 and Table 12 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.

---

**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 RAS Extension Pyro V2 Assays”, page 48).

**Note:** Additional rare or unexpected mutations may be present in the sequenced region, and could be analyzed using alternative “Sequence to Analyze”, considering unexpected mutations. If a Pyrogram trace cannot be aligned with one of the provided “Sequence to Analyze”, the pattern simulation tool in Table 11 and Table 12 can help identify rare mutations by simulating the expected pattern of an unknown mutation. The pattern simulation tool can be obtained from QIAGEN Technical Service.

**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.

### **Re-running samples for detection of low-level mutations**

We strongly recommend that the unmethylated control DNA is included in every run for comparison, and as a control for background levels. Samples showing a mutation frequency only slightly higher than the corresponding position in the control sample, should be examined in relation to the table showing the limit of detection (see Table 9, page 40). If using the RAS Extension Plug-in Report, this is performed automatically. As a guide, samples that have a suspected mutation in the range from LOD (Table 9) to LOD + 3 percentage units, should be re-run in duplicate together with a sample with unmethylated control DNA. If using the RAS Extension Plug-in Report (step 5), a warning will be issued if this occurs. The result of both duplicates should be  $\geq$  LOD and different from the control sample. Otherwise, the sample should be judged as “No Mutation Detected”.

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**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:** The frequencies of mutations as given by the PyroMark software and in the "AQ Analysis Results" report do not necessarily reflect the percentage of mutation.

**Table 9. LOD determined for specific mutations**

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

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

\* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at [www.sanger.ac.uk/genetics/CGP/cosmic](http://www.sanger.ac.uk/genetics/CGP/cosmic).

† Lowest mutation level in a sample resulting in a measured frequency  $\geq$ LOD.

## Representative results

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

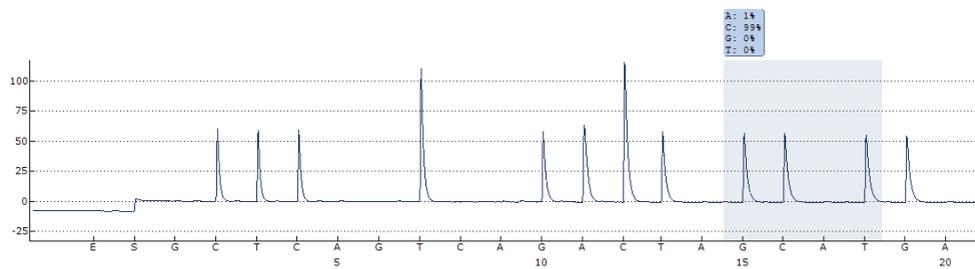


Figure 8. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the KRAS 59/61 assay.

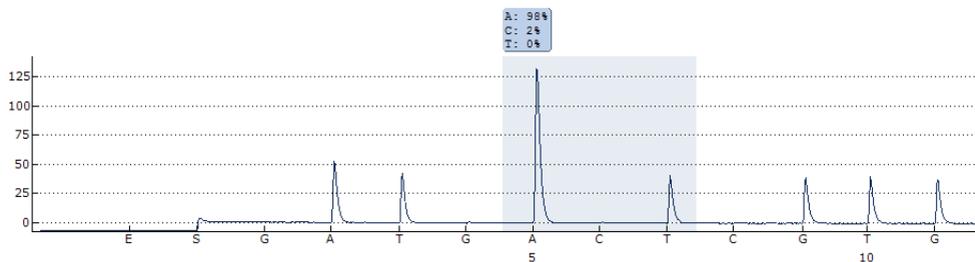


Figure 9. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the KRAS 117 assay.

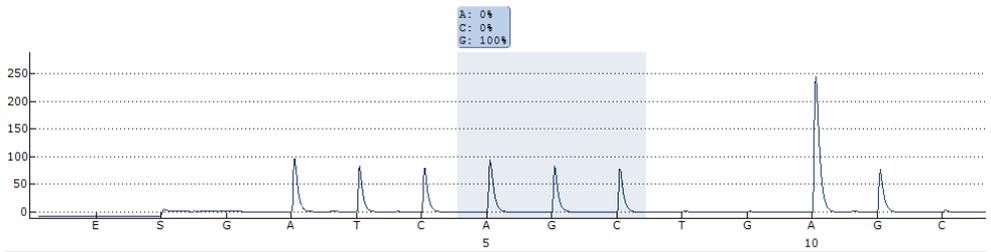


Figure 10. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the KRAS 146 assay.

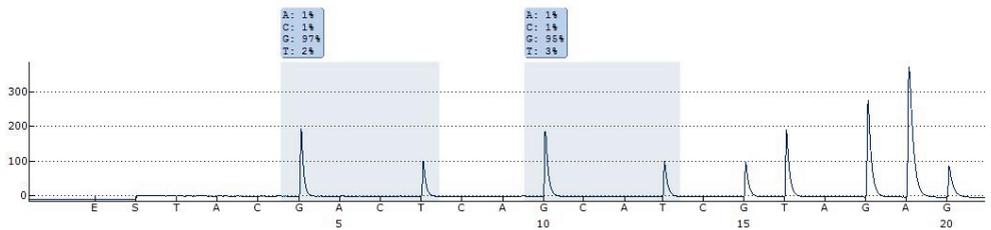


Figure 11. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 12/13 assay.

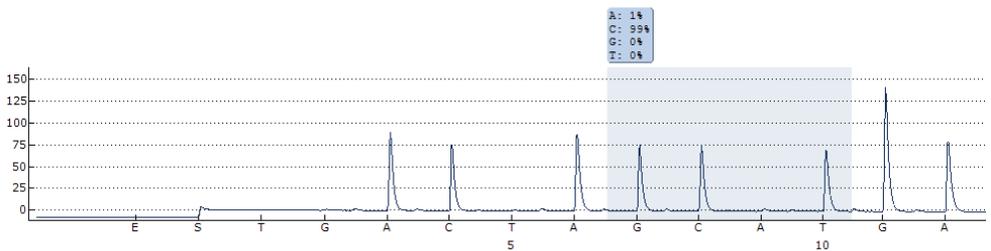


Figure 12. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 59 assay.

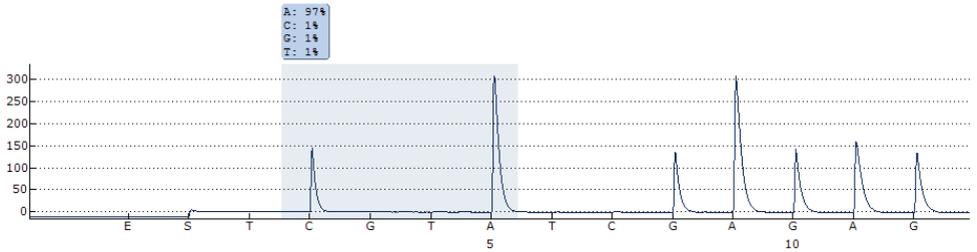


Figure 13. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 61 assay.

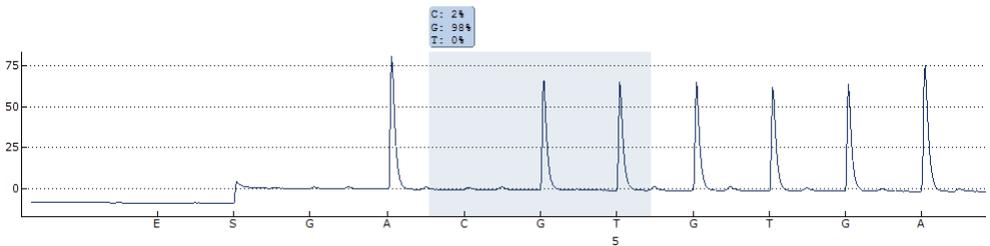


Figure 14. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 117 assay.

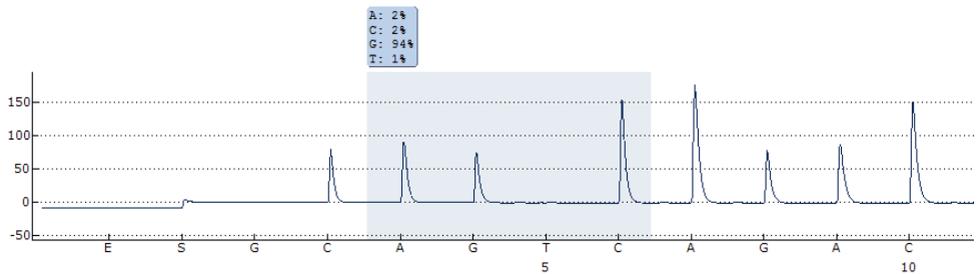


Figure 15. Pyrogram trace obtained after analysis of a sample with a wild-type genotype with the NRAS 146 assay.

# 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](http://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](http://www.qiagen.com)).

Refer to the *PyroMark Q24 User Manual* for general troubleshooting of the instrument.

## Comments and suggestions

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### “Check” or “failed” result

- |  |   |
|--|---|
| a) Low peak height                               | <p>Handling errors in PCR setup or sample preparation prior to Pyrosequencing can result in low peaks.</p> <p>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.</p> <p>Perform the function test for filter probes as described in the <i>PyroMark Q24 User Manual</i> on a regular basis, and exchange filter probes when indicated.</p> <p>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.</p> |
| b) Mutation not defined in “Sequence to Analyze” | <p>Adjust the “Sequence to Analyze” in the assay setup (see “Appendix A: Setting Up RAS Extension Pyro V2 Assays”, page 48), and reanalyze the run. Mutations that are not covered with “Sequences to Analyze” can be identified with the pattern simulation tool.</p>  |
| c) Unexpected rare mutation                      | <p>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.</p>   |

### Comments and suggestions

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- 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.

#### 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 31.
- 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 RAS Extension Pyro V2 Kit is tested against predetermined specifications to ensure consistent product quality.

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## Contact Information

For technical assistance and more information, please see our Technical Support Center at [www.qiagen.com/Support](http://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](http://www.qiagen.com)).

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# Appendix A: Setting Up RAS Extension Pyro V2 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](http://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 RAS Extension Pyro V2 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 10 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 34).

- To check if mutations are present in other nucleotides, change the “Sequence to Analyze” according to Table 10. 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.

- Manually enter the assay specific “Dispensation Order” from Table 10.

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

- Click the “Analysis Parameters” tab, and increase “Peak Height Threshold - Required peak height for Passed quality:” to 30.
- 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 10. Assay setup: “Sequence to analyze” and “Dispensation order” for the eight assays of the RAS Extension Pyro V2 Kit**

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	ABTGTGATT	GACGTGTGA
NRAS 146	CANCCAAGACCA	GCAGTCAGAC

**Table 11. Common mutations in the human KRAS gene detected by the RAS Extension Pyro V2 Kit with respective "Sequence to analyze"**

Nucleic acid substitution	Amino acid substitution	Sequence to analyze	Cosmic ID (V70)*
<b>KRAS codon 59 (GCA)</b>			
175G>A	A59T	CTCTTGACCTGNTGT	546
176C>G	A59G	CTCTTGACCTNCTGT	28518
<b>KRAS codon 61 (CAA)</b>			
183A>C	Q61H	CTCDTGACCTGCTGT	554
182A>T	Q61L	CTCTHGACCTGCTGT	553
182A>G	Q61R	CTCTHGACCTGCTGT	552
183A>T	Q61H	CTCDTGACCTGCTGT	555
181C>G	Q61E	CTCTTSACCTGCTGT	550
<b>KRAS codon 117 (AAA)</b>			
351A>C	K117N	ATAAHTGTGA	19940
351A>T	K117N	ATAAHTGTGA	28519
<b>KRAS codon 146 (GCA)</b>			
436G>A	A146T	ATCAVCAAAGA	19404
436G>C	A146P	ATCAVCAAAGA	19905
437C>T	A146V	ATCAGBAAAGA	19900

\* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at [www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/).

**Table 12. Common mutations in the human NRAS gene detected by the RAS Extension Pyro V2 Kit with respective "Sequence to analyze"**

Nucleic acid substitution	Amino acid substitution	Sequence to analyze	Cosmic ID (V70)*
<b>NRAS codon 12 (GGT)</b>			
34G>A	G12S	NGTNGTGTTGGGAAAAGC	563
34G>T	G12C	NGTNGTGTTGGGAAAAGC	562
34G>C	G12R	NGTNGTGTTGGGAAAAGC	561
35G>A	G12D	GNTGNTGTTGGGAAAAGC	564
35G>T	G12V	GNTGNTGTTGGGAAAAGC	566
35G>C	G12A	GNTGNTGTTGGGAAAAGC	565
<b>NRAS codon 13 (GGT)</b>			
37G>A	G13S	NGTNGTGTTGGGAAAAGC	571
37G>T	G13C	NGTNGTGTTGGGAAAAGC	570
37G>C	G13R	NGTNGTGTTGGGAAAAGC	569
38G>A	G13D	GNTGNTGTTGGGAAAAGC	573
38G>T	G13V	GNTGNTGTTGGGAAAAGC	574
38G>C	G13A	GNTGNTGTTGGGAAAAGC	575
<b>NRAS codon 59 (GCT)</b>			
175G>A	A59T	ACAVCTGGAC	578
176C>G	A59G	ACAGNTGGAC	–
<b>NRAS codon 117 (AAG)</b>			
351G>C	K117N	ABTGTGATTT	–
351G>T	K117N	ABTGTGATTT	–
<b>NRAS codon 61 (CAA)</b>			
181C>A	Q61K	VAAGAAGAGTA	580
182A>G	Q61R	CNAGAAGAGTA	584
182A>T	Q61L	CNAGAAGAGTA	583
183A>T	Q61H	CANGAAGAGTA	585
183A>C	Q61H	CANGAAGAGTA	586
183A>G	Q61Q	CANGAAGAGTA	587

Nucleic acid substitution	Amino acid substitution	Sequence to analyze	Cosmic ID (V70)*
<b>NRAS codon 146 (GCC)</b>			
436G>A	A146T	CANCCAAGACCA	27174
436G>C	A146P	CANCCAAGACCA	–
437C>T	A146V	CAGBCAAGACCA	–

\* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at [www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/).

## Appendix B: Emptying the Waste Container and Troughs

 <p>WARNING</p>	<p>Hazardous chemicals</p> <p>The Denaturation Solution used with the vacuum workstation contains sodium hydroxide, which is irritating to eyes and skin.</p> <p>Always wear safety glasses, gloves, and a lab coat.</p> <p>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.</p> <p>Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.</p>
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\* 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.

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## 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

<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
RAS Extension Pyro V2 Kit (24)	For 24 reactions: Sequencing Primers, PCR Primers, Unmethylated Control DNA, PyroMark PCR Master Mix, CoralLoad Concentrate, Buffers, and Reagents	970590
PyroMark Q24	Sequence-based detection platform for Pyrosequencing of 24 samples in parallel	9001514
PyroMark Q24 Vacuum Workstation	Vacuum Workstation for preparing 24 samples in parallel, from PCR product to single-stranded template	9001516
PyroMark Q24 Software	Analysis software	9019062
<b>Accessories</b>		
PyroMark Q24 Plate (100)	24-well sequencing reaction plate	979201
PyroMark Q24 Cartridge (3)	Cartridges for dispensing nucleotides and reagents	979202
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	979203
PyroMark Q24 Validation Oligo	For performance confirmation of system	979204

Product	Contents	Cat. no.
<b>Related products</b>		
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
EZ1 DNA Tissue Kit (48)	For 48 preps: Reagent Cartridges (Tissue), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 ml), Elution Tubes (1.5 ml), Buffer G2, Proteinase K	953034
PAXgene Tissue Containers (10)	For collection, fixation, and stabilization of 10 samples: 10 Prefilled Reagent Containers, containing PAXgene Tissue FIX and PAXgene Tissue STABILIZER	765112
PAXgene Tissue DNA Kit (50)	For 50 DNA preps: PAXgene DNA Mini Spin Columns, Processing Tubes, Microcentrifuge Tubes, Carrier RNA, and Buffers; to be used in conjunction with PAXgene Tissue Containers	767134

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