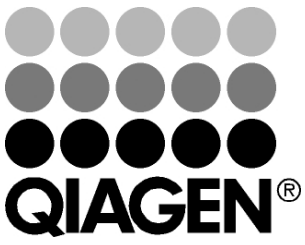
September 2010

# KRAS Pyro® Handbook

For quantitative measurements of mutations in codons 12, 13, and 61 of the human KRAS gene




Sample & Assay Technologies

# QIAGEN Sample and Assay Technologies

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

**QIAGEN sets standards in**:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit www.qiagen.com.

# Contents

# Kit Contents

### KRAS Pyro Kit (box 1/2)

| **KRAS Pyro Kit** | **(24)** |
|---|---|
| **Catalog no.** | **970460** |
| **Number of reactions** | **24** |
| Seq Primer KRAS 12/13 | 24 µl |
| Seq Primer KRAS 61 | 24 µl |
| PCR Primer KRAS 12/13 | 24 µl |
| PCR Primer KRAS 61 | 24 µl |
| PyroMark® PCR Master Mix, 2x | 850 µl |
| CoralLoad® Concentrate, 10x | 1.2 ml |
| $H_2O$ | 3 x 1.9 ml |
| Unmethylated Control DNA, 10 ng/µl | 100 µl |

**Buffers and Reagents (box 2/2)**

| Buffers and Reagents | |
|---|---|
| PyroMark Binding Buffer | 10 ml |
| PyroMark Annealing Buffer | 10 ml |
| PyroMark Denaturation Solution* | 250 ml |
| PyroMark Wash Buffer, 10x | 25 ml |
| Enzyme Mixture | 1 vial |
| Substrate Mixture | 1 vial |
| dATPαS | 1180 µl |
| dCTP | 1180 µl |
| dGTP | 1180 µl |
| dTTP | 1180 µl |
| Handbook | 1 |

* Contains sodium hydroxide.

# Shipping and Storage

The KRAS Pyro Kit is shipped in two boxes. The KRAS 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 5 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 3 freeze–thaw cycles.

**Important**: Nucleotides should not be frozen.

The KRAS Pyro Kit is stable until the kit expiration date when stored under these conditions.

## Product Use Limitations

The KRAS Pyro 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 to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

## Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the KRAS Pyro Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

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

## Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

# Safety Information

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

### 24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

# Quality Control

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

# Introduction

The KRAS Pyro Kit is used for quantitative measurements of mutations in codons 12, 13, and 61 of the human KRAS gene. The product consists of 2 assays: one for detecting mutations in codons 12 and 13 and the second for detecting mutations in codon 61 (Figure 1). The two regions are amplified separately by PCR and sequenced through the defined region. Sequences surrounding the defined positions serve as normalization and reference peaks for quantification and quality assessment of the analysis.

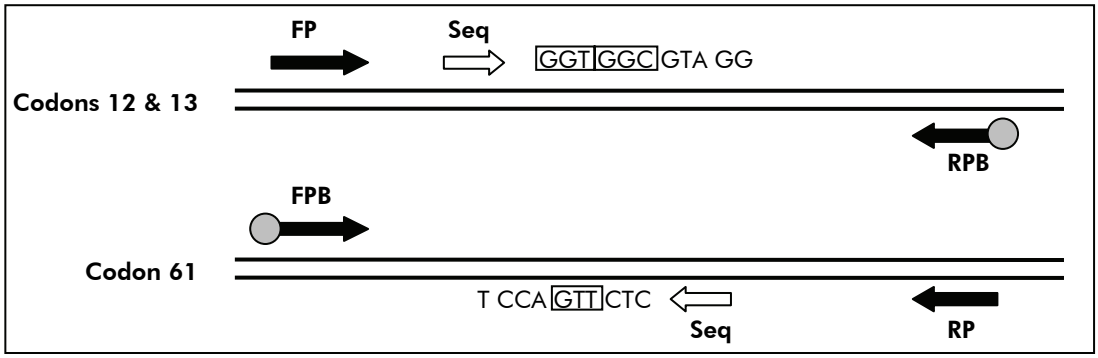



**Figure 1. Illustration of the KRAS assay.** The sequence indicated is the analyzed sequence for a normal sample. **FP** and **FPB**: Forward PCR primers (B indicates biotinylation); **RP** and **RPB**: Reverse PCR primers (B indicates biotinylation); **Seq**: Sequencing primers.

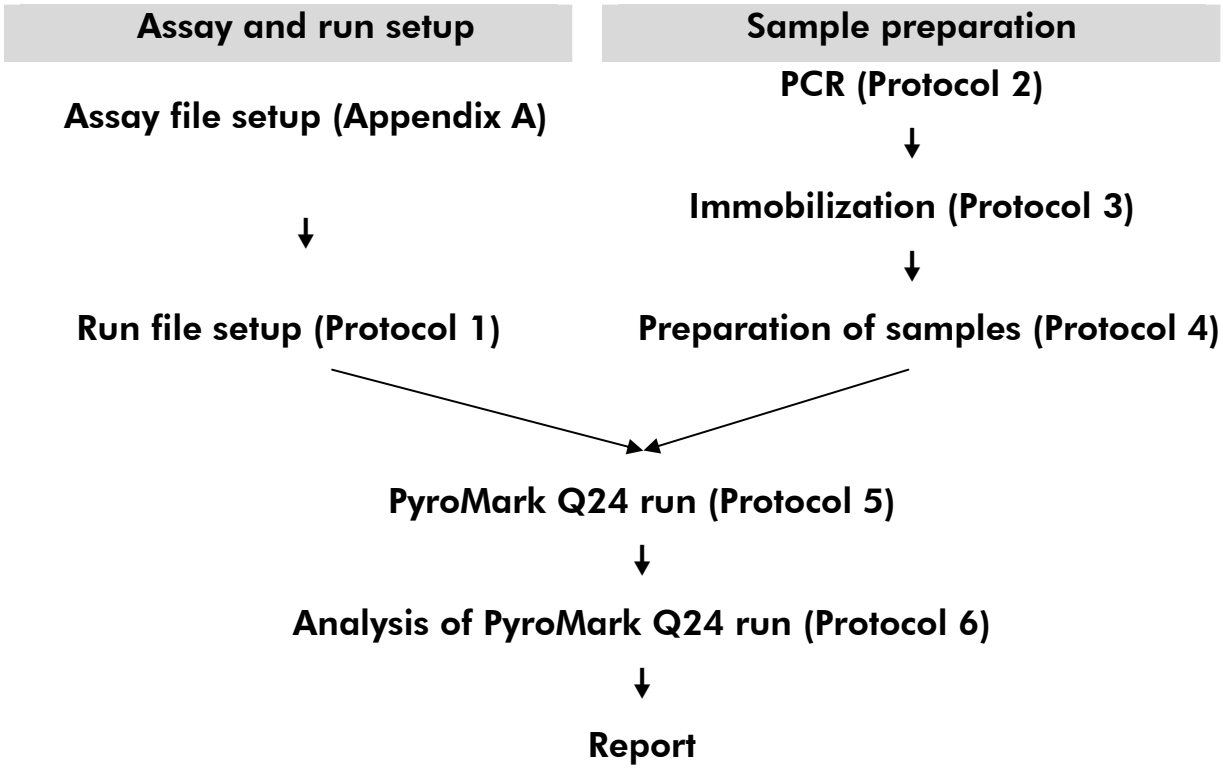
Codons 12 and 13 are sequenced in the forward direction; codon 61 in the reverse direction.

The product consists of a PCR primer mix and a sequencing primer for each assay. The primers are delivered in solution. Each vial contains 24 µl of each primer or primer mix.

# Principle and procedure

The workflow illustrates the assay procedure. After PCR using primers targeting codons 12/13 and codon 61, 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 System using a run setup file and a run file. The KRAS Plug-in Report should be used to analyze the run. However, the run can also be analyzed using the analysis tool integral to the PyroMark Q24 System. The "Sequence to Analyze" can be then adjusted for detection of rare mutations after the run (see "Protocol 6: Analysis of a PyroMark Q24 Run", page 25, and Appendix A, page 33).

**Workflow of KRAS Pyro procedure**



## Equipment and Reagents to Be Supplied by User

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

- DNA isolation kit (see "DNA isolation", page 11)
- Pipets (adjustable)
- Sterile pipet tips with filters (for PCR setup)
- Benchtop microcentrifuge
- Thermal cycler and appropriate PCR tubes
- Streptavidin Sepharose High Performance (GE Healthcare, cat. no. 17-5113-01; www.gelifesciences.com)
- PyroMark Q24 (cat. no. 9001514)
- PyroMark Q24 Software (cat. no. 9019062)
- PyroMark Q24 Plate (cat. no. 979201)
- PyroMark Q24 Cartridge (cat. no. 979202)
- PyroMark Q24 Vacuum Workstation (cat. no. 9001518 [220V] or 9001516 [110V] or 9001519 [100V])
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80°C
- 24-well PCR plate or strips
- Strip caps
- High-purity water (Milli-Q® 18.2 MΩ x cm or equivalent). **Note**: Sufficient water is supplied to dissolve the enzyme mixture and the substrate mixture and also for the PCR and immobilization to Sepharose beads (Protocols 2 and 3). Additional high-purity water is required to dilute PyroMark Wash Buffer, 10x
- Ethanol (70%)

# Important Notes

## General precautions

The user should always pay attention to the following:

- Strict compliance with the user manual is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended, and will result in a loss of performance.
- 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.

## Sample material

All samples must be treated as potentially infectious material.

Specimen material is human DNA extracted from blood or formalin-fixed, paraffin-embedded samples.

Samples from humans undergoing heparin treatment must not be used. Blood samples that have been collected in tubes containing heparin as an anticoagulant should not be used. Heparin affects the PCR.

## DNA isolation

The QIAGEN kits shown in Table 1 (page 12) are recommended for DNA purification from the indicated human sample types for use with the KRAS Pyro Kit. Carry out the DNA purification according to the instructions in the kit handbooks.

**Table 1. DNA purification kits recommended for use with the KRAS Pyro Kit**

| Sample material | 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 |
| Blood | QIAamp DSP DNA Blood Mini Kit | 61104 |

* Following the protocol for use with paraffin-embedded tissue. The EZ1 DNA Tissue Kit should 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).

## Controls

Unmethylated control DNA is included in the product as a positive control for PCR and sequencing reactions.

In addition, a negative control (without template DNA) should always be included.

# Protocol 1: Run Setup for the PyroMark Q24 System

### Things to do before starting

- If the KRAS Plug-in Report has not been installed, create an Assay Setup as described in Appendix A. The KRAS Plug-in Report can be obtained by e-mail from pyro.plugin@qiagen.com. This must only be done once, before running the KRAS Pyro assay for the first time (see Appendix A, page 33).

### Procedure

1. **Click in the toolbar.**

   A new run file is created.

2. **Enter the run parameters (see "Run parameters", page 14).**
3. **Set up the plate by adding assays for both codons 12/13 and codon 61 to wells corresponding to the samples to analyze. A negative control sample (without DNA), and the unmethylated control DNA provided are recommended as controls.**
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 and, when the report appears, click .**
5. **Close the run file and copy it to a USB stick (supplied with the system) using Windows® Explorer.**

   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 18).

   To run the plate on PyroMark Q24 System, see "Protocol 5: Running the PyroMark Q24 System", page 23.

### 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 reagents and 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 KRAS Pyro Kit to be used. The lot number can be found on the product label.<br>We recommend entering both the reagent ID and the kit ID so that any unexpected problems with the reagents 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 KRAS Pyro Kit

This protocol is for PCR amplification of a region containing codon 12 and codon 13, and a separate PCR amplification of a region containing codon 61 using the KRAS Pyro Kit.

### Important points before starting

- The HotStarTaq® DNA Polymerase in the PyroMark Master Mix requires an activation step of **15 min at 95°C**.
- Set up all reaction mixtures in an area separate from that used for DNA purification, adding template DNA 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 reagents.**
   Mix well before use.
2. **Prepare a reaction mix for each PCR primer set according to Table 2.**
   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 2. Preparation of reaction mix for each PCR primer mix**

| Component | Volume/reaction |
|---|---|
| PyroMark PCR Master Mix, 2x | 12.5 µl |
| CoralLoad Concentrate, 10x | 2.5 µl |
| PCR Primer KRAS 12/13 **or**<br>PCR Primer KRAS 61 | 1 µl |
| Water (supplied) | 4 µl |
| **Total volume** | **20 µl** |

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 3), and mix thoroughly.**

   A negative control (without template DNA) should always be included.

   Include a reaction with unmethylated control DNA as positive control (see "Controls", page 12).

**Table 3. Preparation of PCR**

| Component | Volume/reaction |
|---|---|
| Reaction mix | 20 µl |
| Sample DNA | 5 µl |
| **Total volume** | **25 µl** |

5. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 4.**

**Table 4. Optimized cycling protocol**

| | | | 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 18.**

# 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 (GE Healthcare) 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.

### 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 5. Prepare a volume 10% greater than that required for the total number of reactions to be performed.**

**Table 5. Master mix for DNA immobilization**

| Component | Volume/sample |
|---|---|
| Streptavidin Sepharose High Performance | 2 µl |
| PyroMark Binding Buffer | 40 µl |
| Water (supplied) | 28 µl |
| **Total volume** | **70 µl** |

3. **Add 70 µl of the master mix to wells of a 24-well PCR plate or strips as predefined in the run setup (see "Protocol 1: Run Setup for the PyroMark Q24 System", page 13).**
4. **Add 10 µl biotinylated PCR product from Protocol 2 to each well containing master mix as predefined in the run setup (see "Protocol 1: Run Setup for the PyroMark Q24 System", page 13).**

   The total volume per well should be 80 µl after addition of the master mix and PCR product.
5. **Seal the PCR plate (or strips) using strip caps.**

   Ensure that no leakage is possible between the wells.

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

   During this step, prepare the PyroMark Q24 Vacuum Workstation for sample preparation, as described in the *PyroMark Q24 User Manual*.

7. **Proceed immediately with "Protocol 4: Preparation of Samples Prior to Pyrosequencing Analysis on the PyroMark Q24 ", page 20.**

   Sepharose beads sediment quickly. Capturing of beads must take place immediately following agitation.

# 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 2 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 13), depending on the region of analysis (codons 12 and 13, or codon 61)

### Things to do before starting

- Place the PyroMark Q24 Plate Holder on a heating block at 80°C for use in step 17.
- 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).

### Procedure

1. **Dilute a sufficient amount of each sequencing primer, Seq Primer KRAS 12/13 and Seq Primer KRAS 61, in PyroMark Annealing Buffer as shown in Table 6.**

   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 + one extra).

**Table 6. Example dilution of the sequencing primers**

| Component | Volume/sample | Volume for 9 + 1 reactions |
|---|---|---|
| Seq Primer KRAS 12/13 **or** Seq Primer KRAS 61 | 0.8 µl | 8 µl |
| PyroMark Annealing Buffer | 24.2 µl | 242 µl |
| **Total volume** | **25 µl** | **250 µl** |

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 ", page 13).**

   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. **Place the PCR plate (or strips) from Protocol 3 and the PyroMark Q24 Plate on the worktable.**

   Ensure that the plate is in the same orientation as when samples were loaded.


**Figure 2. Placement of PCR plate (or strips) and PyroMark Q24 plate on the vacuum workstation.**

4. **Apply vacuum to the tool by opening the vacuum switch.**
5. **Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool.**

   Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.

6. **Transfer the tool to the trough containing 40 ml 70% ethanol (trough 1). Flush the filter probes for 5 s.**
7. **Transfer the tool to the trough containing 40 ml Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
8. **Transfer the tool to the trough containing 50 ml Wash Buffer (trough 3). Flush the filter probes for 10 s.**
9. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes.**

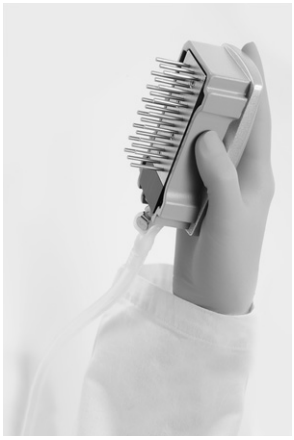


**Figure 3. Illustration of the vacuum tool raised to beyond 90° vertical.**

10. **While the tool is held over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).**
11. **Release the beads in the plate containing the Seq Primers by shaking the tool gently from side to side.**
12. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
13. **Wash the filter probes by lowering the probes into high-purity water (trough 5) and applying vacuum. Flush the probes with 70 ml high-purity water.**
14. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes.**
15. **Close the vacuum switch on the tool (Off), and place the tool in the Parking (P) position.**
16. **Turn off the vacuum pump.**

    At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage, see Appendix B, page 35.
17. **Heat the PyroMark Q24 Plate with the samples at 80°C for 2 min using the prewarmed PyroMark Q24 Plate Holder.**
18. **Remove the PyroMark Q24 Plate from the plate holder, and allow the samples cool to room temperature (15–25°C) for 5–10 min.**
19. **Proceed with "Protocol 5: Running the PyroMark Q24 System", page 23.**

# Protocol 5: Running the PyroMark Q24 System

This protocol describes the loading of PyroMark Gold 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 point 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 13), provides information about the volume of nucleotides, enzyme, and substrate buffer needed for a specific assay.

### Procedure

1. **Dissolve each of the freeze-dried enzyme and substrate mixtures in 620 µl water (supplied).**
2. **Load the PyroMark Q24 Cartridge with the appropriate volumes of nucleotides, enzyme, and substrate mixes.**
3. **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.**
4. **Ensure the line is visible in front of the cartridge and close the gate.**
5. **Open the plate-holding frame and place the plate on the heating block.**
6. **Close the plate-holding frame and the instrument lid.**
7. **Insert the USB stick (containing the run file) into the USB port at the front of the instrument.**

   Do not remove the USB port before the run is finished.
8. **Select "Run" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".**
9. **Select the run file using the ▲ and ▼ screen buttons.**

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

15. **Close the gate.**
16. **Open the plate-holding frame and remove the plate from the heating block.**
17. **Close the plate-holding frame and the instrument lid.**
18. **Discard the plate and clean the cartridge, as per the instructions in the product sheet supplied with the cartridge.**
19. **Analyze the run according to "Protocol 6: Analysis of a PyroMark Q24 Run", page 25.**

# Protocol 6: Analysis of a PyroMark Q24 Run

This protocol describes the mutation analysis of a finished KRAS run using PyroMark Q24 Software.

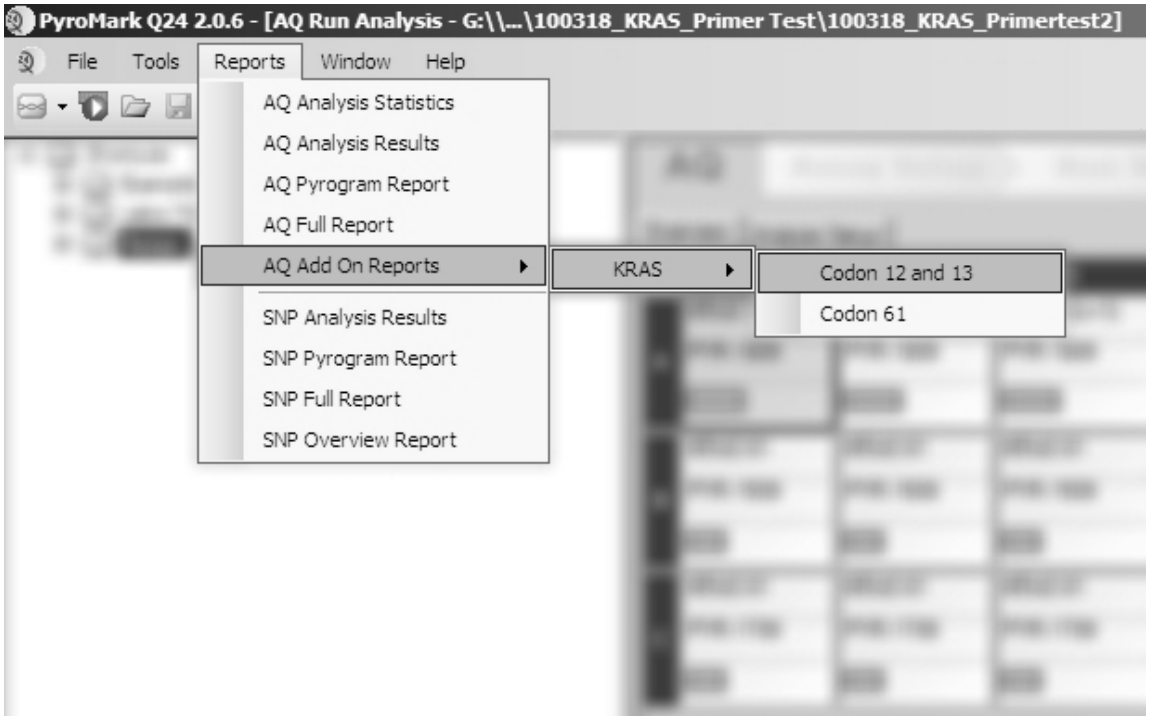
**Procedure**

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 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 2 methods for analyzing the run. If using the KRAS Plug-in Report, go to step 5. If using the AQ analysis integral to the PyroMark Q24 System, go to step 6.**

   **Note**: We strongly recommend using the KRAS Plug-in Report. This report ensures that the correct LODs are used and different sequences to analyze are automatically used to detect all mutations.

5. **Using the KRAS Plug-in Report:**

   **To generate a report, select "AQ Add On Reports/KRAS" and "Codon 12 and 13" or "Codon 61" from "Reports" in the menu.**



**The wells will automatically be analyzed for all mutations for which LOD is given in Table 7 (page 28). The results will be presented in an overview table (see example below), followed by the detailed results containing e.g., Pyrograms and analysis quality.**

**Summary**

| Well | Sample ID | Result | Frequency | Codon Change | Amino Acid Substitution | Info |
|---|---|---|---|---|---|---|
| A1 | 13 | Wildtype | | | | ! |
| A2 | 14 | Wildtype | | | | |
| A3 | 15 | Wildtype | | | | |
| A4 | 16 | Mutation (codon 12) | 4,2 % | GGT>AGT | G12S | ! |
| B2 | 18 | Wildtype | | | | |
| B3 | 19 | Wildtype | | | | |
| B4 | 20 | Mutation (codon 12) | 4,6 % | GGT>AGT | G12S | ! |
| C1 | 21 | Wildtype | | | | |
| C2 | 22 | Wildtype | | | | |
| C3 | 38 | Mutation (codon 12) | 29,7 % | GGT>GAT | G12D | |
| C4 | 39 | Mutation (codon 13) | 42,5 % | GGC>GAC | G13D | |

! See detailed results for further explanation.

6. **Using the 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*.

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

   **Note**: The most frequent mutations in KRAS are found at nucleotide 35 (second base of codon 12). Therefore, the standard "Sequence to Analyze" for the KRAS Codon 12 and 13 assay as defined in the Analysis Setup addresses mutations at this position (see Appendix A, page 33). If a sample contains a mutation at nucleotide 34 (first base of codon 12) the "Sequence to Analyze" can be changed to analyze also the mutation status at this position, as described in Appendix A. Similarly, the "Sequence to Analyze" can be changed for the KRAS Codon 61 assay, as described in Appendix A.

   Updated frequencies of mutations in the human KRAS gene in codon 12/13 and codon 61 are provided online by the Sanger Institute at www.sanger.ac.uk/genetics/CGP/cosmic/.

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 (see Appendix A and the *PyroMark Q24 User Manual*).

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

### Reanalysis of samples with no mutation detected in nucleotide 35 (Codon12) or 183 (Codon 61) or with "Check" or "Failed" quality assessment.

We strongly recommend reanalyzing all samples with no mutation detected with the standard "Sequence to Analyze", as well as samples that received a "Check" or "Failed" quality assessment. "Check" and "Failed" quality assessments may indicate a mutation in a position other than nucleotide 35 or 183, resulting in peak height deviations at reference dispensations. For example, a peak in any of the first 3 dispensations shows that a mutation is present at nucleotide 34.

To reanalyze and target mutations at nucleotide 34, go to "Analysis Setup" and change "Sequence to Analyze" from ***GNT****GRCGTAGGC* to ***NGT****GRCGTAGGC*. Click "Apply", and then click "To All" when the "Apply Analysis Setup" window appears.

To reanalyze and target mutations at nucleotide 182 (second position of Codon 61), change the "Sequence to Analyze" of the Codon 61 assay to the following sequence:
*CTC****THG****ACCTG*

To reanalyze and target mutations at nucleotide 181 (first position of Codon 61), change the "Sequence to Analyze" of the Codon 61 assay to the following sequence:
*CTC****TTS****ACCTG*

**Note**: Ensure the threshold for single peak height is set to 30 RLU.

### Rerunning samples for detection of low-level mutations

It is strongly recommended that a wild-type control sample is included in every run for comparison. Any sample showing a mutation frequency higher than the corresponding position in the wild-type control sample should be examined in relation to the table showing the limit of detection (see Table 7, page 28). If using the KRAS Plug-in Report, this is performed automatically.

As a guide, samples that have a suspected mutation in the range from LOD (Table 7) to LOD + 3 % units should be reanalyzed in duplicate together with a wild-type control sample in duplicate. If using the Plug-in Report (step 5), a warning will be issued if this occurs. If both duplicates give the same result as

the original analysis and are visibly different from the wild-type control, then the sample can be considered to be positive for the mutation.

In case of a suspected GGT → GTT mutation, a result greater than 1% can be considered positive. This level may vary considerably between replicates.

**Table 7. LOD determined for specific mutations**

| Mutation | LOD (% units) | COSMIC ID* (V42) |
|---|---|---|
| **Codon 12 (GGT)** | | |
| GAT | 2.2 | 521 |
| GTT | 1.0 (7†) | 520 |
| TGT | 2.1 | 516 |
| AGT | 1.9 | 517 |
| GCT | 2.3 | 522 |
| CGT | 1.8 | 518 |
| **Codon 13 (GGC)** | | |
| GAC | 1.9 | 532 |
| **Codon 61 (CAA), as assayed in reverse orientation (TTG)** | | |
| GTG | 2.8 | 554 |
| TAG | 3.1 | 553 |
| TCG | 3.5 | 552 |
| ATG | 2.6 | 555 |
| TTC | 3.1 | 550 |

* 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. For further explanation, refer to the text above.

The KRAS Plug-in Report algorithm was used to generate the LOD data. Manual analysis as described in Protocol 6 (page 25) may result in slightly different values.

## Representative results using the AQ analysis integral to the PyroMark Q24 System

Representative Pyrogram results are shown in Figures 4–8.

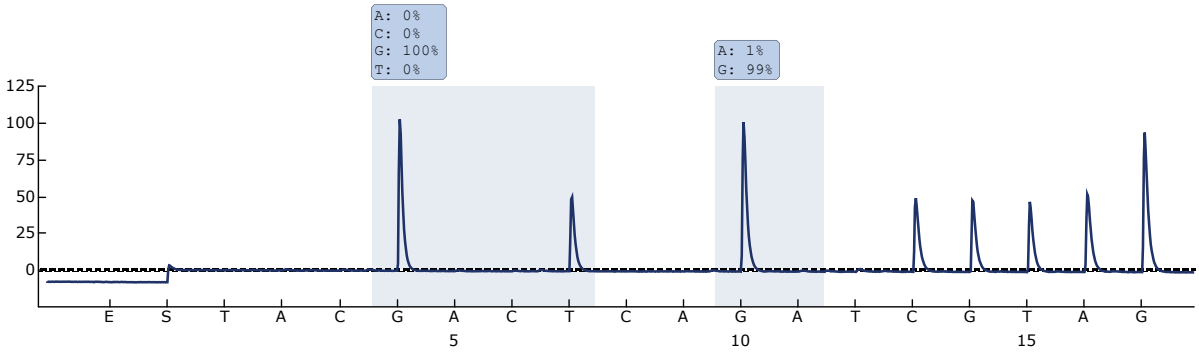



**Figure 4. Pyrogram trace obtained after analysis of a sample with a normal genotype in codons 12 and 13.**

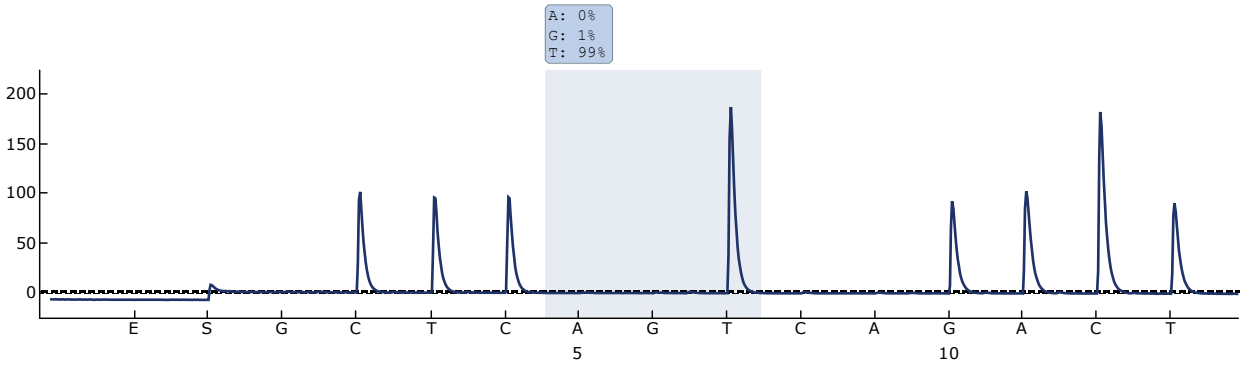



**Figure 5. Pyrogram trace obtained after analysis of a sample with a normal genotype in codon 61.**

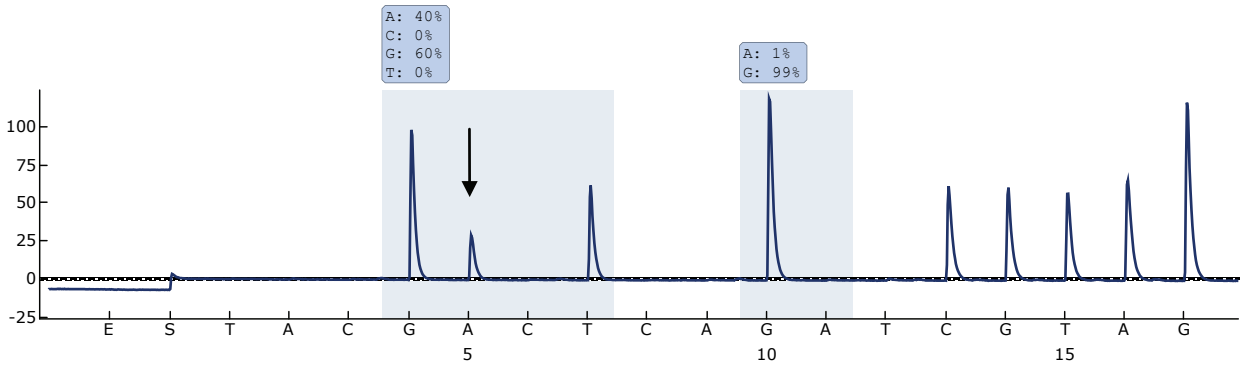



**Figure 6. Pyrogram trace obtained after analysis of samples with a GGT → GAT mutation in base 2 of codon 12 (nucleotide 35, indicated with an arrow).**

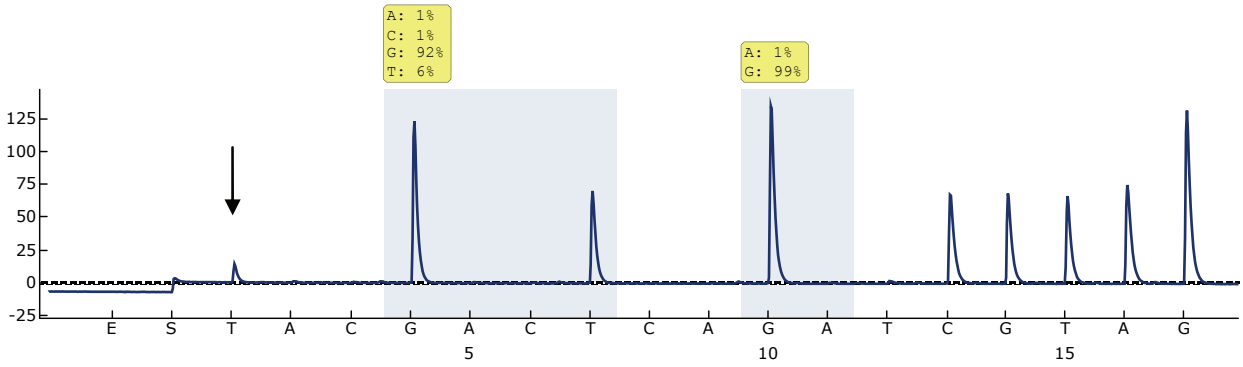



**Figure 7. Pyrogram trace obtained after analysis of samples with a GGT → TGT mutation in base 1 of codon 12 (nucleotide 34, indicated with an arrow) with the "Sequence to Analyze" *GNTGRCGTAGGC* targeting base 2 in codon 12 (nucleotide 35).** A yellow color indicates that this sequence is unexpected and needs to be checked.

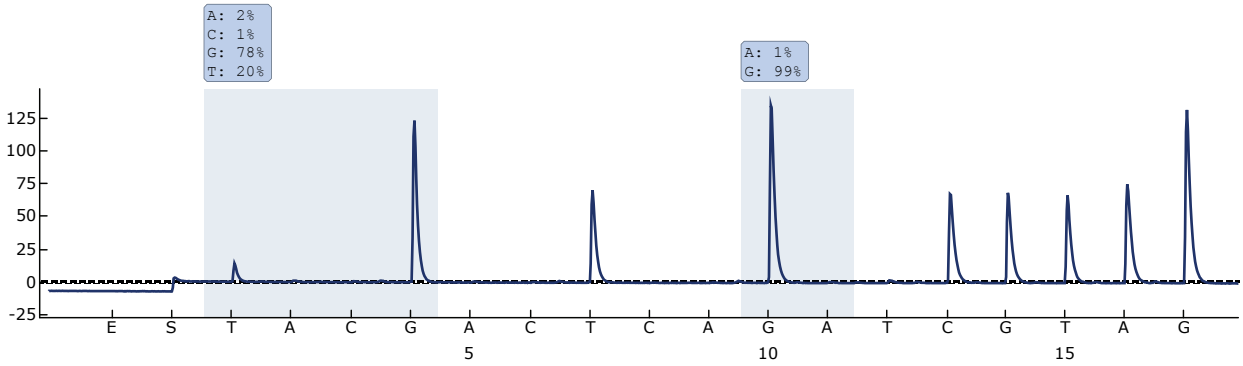



**Figure 8. Pyrogram trace and result obtained after reanalysis of the sample in Figure 7.** The mutation GGT → TGT was reanalyzed with the "Sequence to Analyze" ***NGTGRCGTAGGC*** targeting base 1 in codon 12 (nucleotide 34).

# 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).

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

| | Comments and suggestions |
|---|---|
| **Signals in the no template control (negative control)** | |
| a) Cross-talk between wells | Signal from one well is detected in a neighboring well. If re-running samples, avoid placing samples with high signal intensities next to no template control wells. |
| b) PCR contamination | Use sterile pipet tips with filters. Store and extract materials such as specimens, plasmid controls, and amplicons separately from PCR reagents. |
| **Poor or unexpected sequence** | |
| a) Low quality genomic DNA | Low-quality genomic DNA can cause the PCR to fail. Analyze PCR samples using an electrophoretic technique (using, for example, the QIAxcel® System or agarose-gel electrophoresis). |
| b) 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 standard "Sequence to Analyze". These samples should be analyzed using the alternative "Sequence to Analyze" considering unexpected mutations. |
| **"Check" or "failed" result** | |
| a) Rare mutation not defined in the assay setup | Adjust the sequence to analyze in the assay setup (see Appendix A, page 33), and reanalyze the run. |

| | Comments and suggestions |
|---|---|
| b) Low peak height | Handling errors in PCR setup or sample preparation prior to Pyrosequencing can result in low peaks. It is recommended to reanalyze the sample. |

**High background**

| | |
|---|---|
| Incorrect storage of nucleotides | Store nucleotides at 2–8°C. Storage at –15 to –25°C can cause an increase in the background. |

**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 PyroMark Q24 Gold Reagents according to the instructions supplied with the reagents. |

# Appendix A: Setting Up KRAS Pyro Assays

If the KRAS Plug-in Report has been installed, use the assay setups supplied for codons 12 and 13 and codon 61. The following steps do not need to be performed. The KRAS Plug-in Report can be obtained from pyro.plugin@qiagen.com. We strongly recommend the use of the KRAS Plug-in Report over manual analysis. After installation of the plug-in or each time new software is installed or upgraded on the office computer, the correct function of the Plug-in should be verified as described in the KRAS Plug-In Quick Guide.

If the KRAS Plug-in Report has not been installed, the assay file must be set up manually before running the KRAS Pyro assay for the first time, as described below.

### Procedure

### KRAS codons 12 and 13

**A1. Set up the assay for KRAS codons 12 (position 2) and 13 (position 2) by using the PyroMark Q24 Software.**

**A2. Click in the toolbar and select "New AQ Assay".**

**A3. Type the following sequence in "Sequence to Analyze": *GNTGRCGTAGGC***

The most frequent mutations in codon 12 will be detected in nucleotide 35 (second position) using this "sequence to Analyze". To analyze if mutations are present in nucleotide 34 (first position), change the "Sequence to Analyze" to the following sequence:
*NGTGRCGTAGGC*

**Note**: Ensure the threshold for single peak height is set to 30 RLU.

**A4. Manually enter the following "Dispensation Order": *TACGACTCAGATCGTAG***

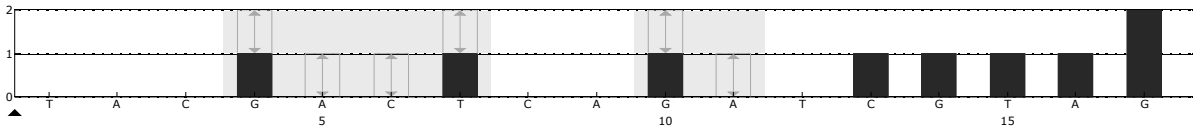



**Figure 9. Histogram for codons 12 (nucleotide 35) and 13 (nucleotide 38) with the "Sequence to Analyze" *GNTGRCGTAGGC*.**

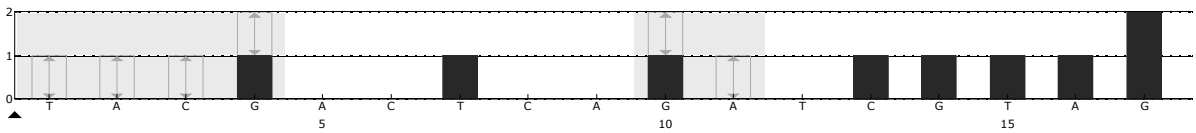



**Figure 10. Histogram for codons 12 (nucleotide 34) and 13 (nucleotide 38) with the "Sequence to Analyze" *NGTGRCGTAGGC*.**

**A5. Click the "Analysis Parameters" tab, and increase "Peak Height Threshold - Required peak height for Passed quality:" to 30.**

**A6. Click in the toolbar, and save the assay as "KRAScodon 12+13".**

**KRAS codon 61**

**A1. Click in the toolbar and select "New AQ Assay".**

**A2. Type the following sequence in "Sequence to Analyze":**
***CTCDTGACCTG***

The most frequent mutations in codon 61 will be detected in nucleotide 183 (third position) with this sequence to analyze. To analyze if mutations are present in nucleotide 182 (second position), change the "Sequence to Analyze" to the following sequence:
*CTCTHGACCTG*

To analyze if mutations are present in nucleotide 181 (first position), change the "Sequence to Analyze" to the following sequence.
*CTCTTSACCTG*

**Note**: Ensure the threshold for single peak height is set to 30 RLU.

**A3. Manually add the following "Dispensation Order".**
***GCTCAGTCAGACT***

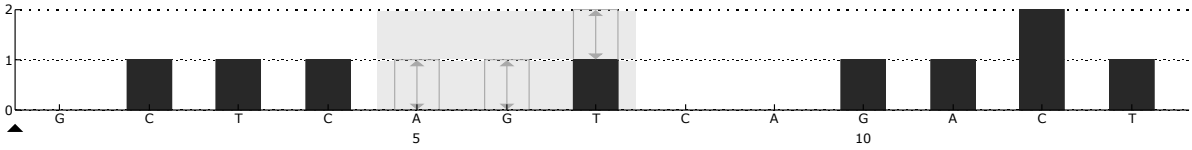



**Figure 11. Histogram for codon 61 (nucleotide 183) with the "Sequence to Analyze" *CTCDTGACCTG*.**

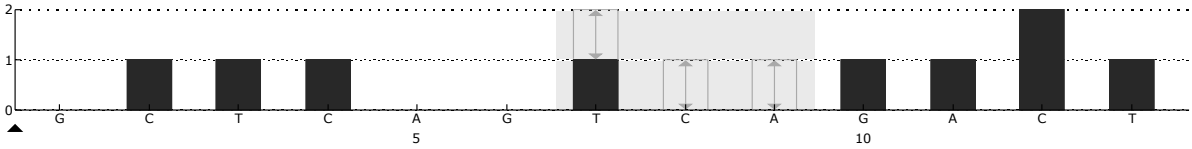



**Figure 12. Histogram for codon 61 (nucleotide 182) with the "Sequence to Analyze" *CTCTHGACCTG*.**

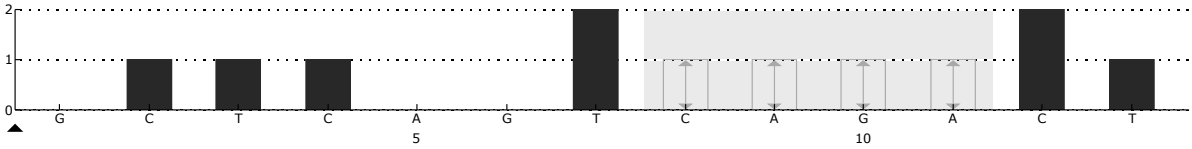



**Figure 13. Histogram for codon 61 (nucleotide 181) with the "Sequence to Analyze" *CTCTTSACCTG*.**

**A4. Click the "Analysis Parameters" tab, and increase "Peak Height Threshold - Required peak height for Passed quality:" to 30.**

**A5. Click in the toolbar, and save the assay as "KRAScodon 61".**

# 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.<br>Always wear safety glasses, gloves, and a lab coat.<br>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 Material Safety Data Sheets (MSDSs) or OSHA,* ACGIH,† or COSHH‡ documents.<br>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 (Milli-Q 18.2 MΩ x cm, www.millipore.com, or equivalent).

### Procedure

**B1. Ensure that no vacuum is applied to the vacuum tool. Make sure that the vacuum is closed (Off) and the vacuum pump is switched off.**

**B2. Discard any solutions left in the troughs.**

**B3. Rinse the troughs with high-purity water, or replace them if necessary.**

**B4. Empty the waste container.**

The cap can be removed without disconnecting the tubing.

**B5. If the vacuum workstation must be cleaned (for example, due to dust or spillage), follow the instructions in the *PyroMark Q24 User Manual*.**

# References

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

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

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| Product | Contents | Cat. no. |
|---|---|---|
| KRAS Pyro Kit (24) | For 24 reactions on PyroMark Q24 Systems: Seq Primers, PCR Primers, Unmethylated Control DNA, PyroMark PCR Master Mix, CoralLoad Concentrate, PyroMark Binding Buffer, PyroMark Annealing Buffer, PyroMark Denaturation Solution, PyroMark Wash Buffer, Enzyme Mixture, Substrate Mixture, dATPαS, dCTP, dGTP, dTTP, and $H_2O$ | 970460 |
| 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 | 9001518 (220 V); 9001516 (110 V); 9001519 (100 V) |
| PyroMark Q24 Software | Analysis software | 9019062 |
| **Accessories** | | |
| 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 |

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| 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 |
| 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 |
| QIAamp DSP DNA Blood Mini Kit | For 50 preps: QIAamp Mini Spin Columns, Buffers, Reagents, Tubes, VacConnectors | 61104 |

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