

September 2010

NRAS Pyro[®] Handbook

For quantitative measurement of mutations in
codons 12, 13, and 61 of the human NRAS gene



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Contents

Kit Contents	4
Shipping and Storage	5
Product Use Limitations	6
Product Warranty and Satisfaction Guarantee	6
Technical Assistance	6
Quality Control	7
Safety Information	7
Introduction	8
Principle and procedure	8
Equipment and Reagents to Be Supplied by User	10
Important Notes	11
General precautions	11
Sample material	11
DNA isolation	11
Controls	12
Protocols	
■ 1: Run Setup for the PyroMark Q24 System	13
■ 2: PCR Using the Reagents Supplied with the NRAS Pyro Kit	15
■ 3: Immobilization of PCR Products to Streptavidin Sepharose High Performance Beads	18
■ 4: Preparation of Samples Prior to Pyrosequencing Analysis on the PyroMark Q24	20
■ 5: Running the PyroMark Q24 System	23
■ 6: Analysis of a PyroMark Q24 Run	25
Troubleshooting Guide	29
Appendix A: Setting Up NRAS Pyro Assays	31
Appendix B: Emptying the Waste Container and Troughs	33
References	35
Ordering Information	36

Kit Contents

NRAS Pyro Kit (box 1/2)

NRAS Pyro Kit (24)	(24)
Catalog no.	970530
Number of reactions	24
Seq Primer NRAS 12/13	24 μ l
Seq Primer NRAS 61	24 μ l
PCR Primer NRAS 12/13	24 μ l
PCR Primer NRAS 61	24 μ l
PyroMark [®] PCR Master Mix, 2x	850 μ l
CoralLoad [®] Concentrate, 10x	1.2 ml
H ₂ O	3 x 1.9 ml
Unmethylated Control DNA, 10 ng/ μ l	100 μ l

Pyro 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
dATP α S	1180 μ l
dCTP	1180 μ l
dGTP	1180 μ l
dTTP	1180 μ l
Handbook	1

* Contains sodium hydroxide.

Shipping and Storage

The NRAS Pyro Kit is shipped in two boxes. The NRAS 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, dATP α S, 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 NRAS Pyro Kit is stable until the kit expiration date when stored under these conditions.

Product Use Limitations

The NRAS 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.

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).

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 NRAS 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).

Quality Control

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

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

Introduction

The NRAS Pyro Kit is used for quantitative measurements of mutations in codons 12, 13, and 61 of the human NRAS gene. The encoded protein is a GTPase.

The kit consists of 2 assays: one for detecting mutations in codons 12 and 13 and the other for detecting mutations in codon 61. 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.

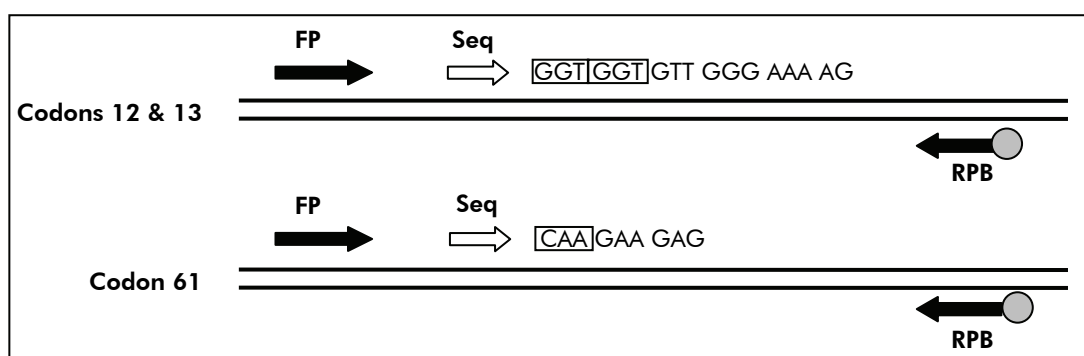


Figure 1. Illustration of the NRAS assay. The sequence indicated is the analyzed sequence for a normal sample. **FP**: Forward PCR primers; **RPB**: Reverse PCR primers (B indicates biotinylation); **Seq**: Sequencing primers.

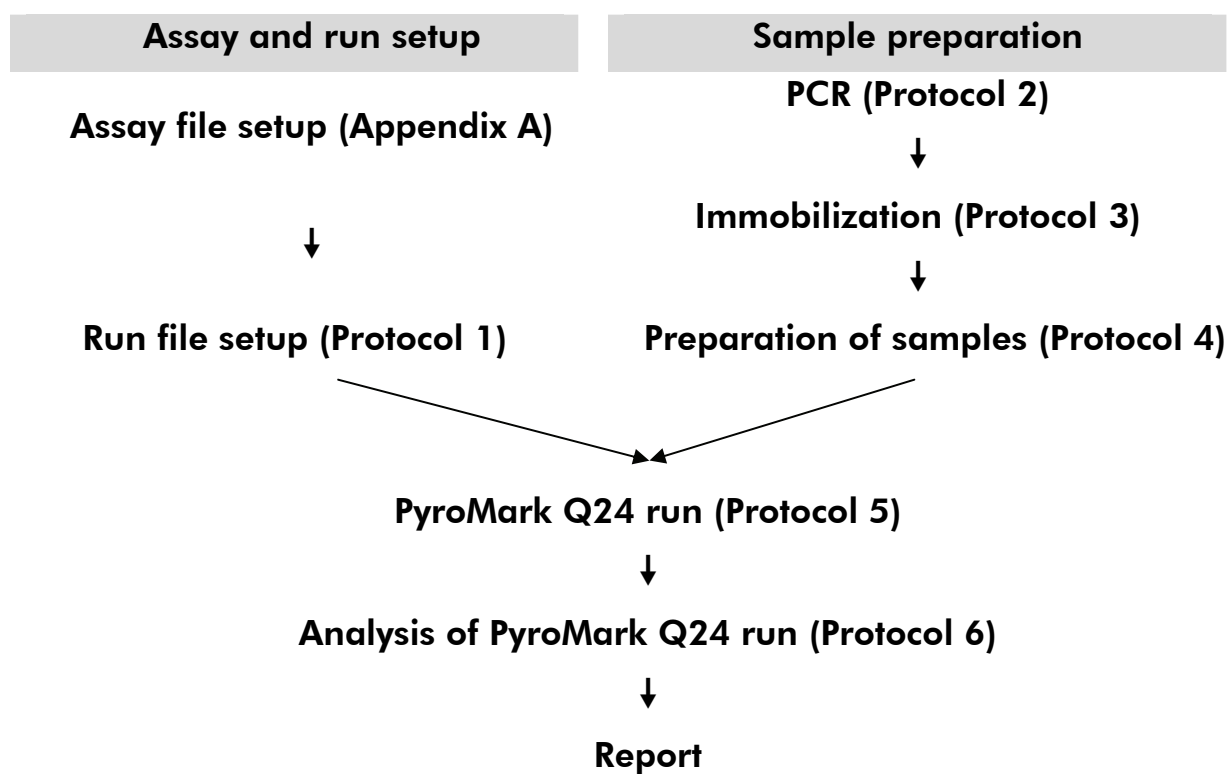
Both assays are sequenced in the forward direction

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

Principle and procedure

The workflow on page 9 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 using a run setup file and a run file. The “Sequence to Analyze” can be adjusted for detection of rare mutations after the run (see “Protocol 6: Analysis of a PyroMark Q24 Run”, page 25, and Appendix A, page 31).

Workflow of NRAS 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 provided in the kit for PCR, to dissolve the Enzyme Mixture and the Substrate Mixture, and for preparation of the master mix for DNA immobilization; 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:

- 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 kits from QIAGEN shown in Table 1 (page 12) are recommended for DNA purification from the indicated human sample types for use with the NRAS 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 NRAS 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 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).

Controls

Unmethylated control DNA is included in the kit 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

Important point before starting

- Create an Assay Setup as described in Appendix A. This needs to be done only once, before running the NRAS Pyro assay for the first time (see Appendix A, page 31).

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 sample (without template 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 . 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 set up (see “Protocol 3: Immobilization of PCR Products to Streptavidin Sepharose High Performance Beads”, page 18).

To run the plate on PyroMark Q24, 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 NRAS Pyro Kit to be used. The lot number can be found on the product label. 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 Reagents Supplied with the NRAS Pyro Kit

This protocol is for PCR amplifications of a region containing codon 12 and codon 13, and a separate PCR amplification of a region containing codon 61 using the NRAS 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, PCR setup, 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 to 0.4–2 ng/μl.

Procedure

1. Thaw all necessary components (see Table 2).

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 μ l
CoralLoad Concentrate, 10x	2.5 μ l
PCR Primer NRAS 12/13 or PCR Primer NRAS 61	1 μ l
Water (supplied)	4 μ l
Total volume	20 μl

3. Mix the reaction mix thoroughly, and dispense 20 μ l 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 μ l template DNA (2–10 ng of genomic DNA) to the individual PCR tubes (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 μ l
Sample DNA	5 μ l
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.

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 μ l
PyroMark Binding Buffer	40 μ l
Water (supplied)	28 μ l
Total volume	70 μl

3. Add 70 μ l 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 μ l of 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 μ l 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, add high-purity water to 25 ml 10x PyroMark Wash Buffer to achieve a final volume of 250 ml and obtain a 1x working solution.

Procedure

1. **Dilute a sufficient amount of each sequencing primer, Seq Primer NRAS 12/13 and Seq Primer NRAS 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 NRAS 12/13 or Seq Primer NRAS 61	0.8 µl	8 µl
PyroMark Annealing Buffer	24.2 µl	242 µl
Total volume	25 µl	250 µl

2. **Add 25 μ l 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 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 (Figure 2).**

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 switching on the vacuum.**
5. **Carefully lower the filter probes of the vacuum tool 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 vacuum 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 vacuum tool to the trough containing 40 ml 70% ethanol (trough 1; Figure 2). Flush the filter probes for 5 s.**
7. **Transfer the vacuum tool to the trough containing 40 ml Denaturation Solution (trough 2; Figure 2). Flush the filter probes for 5 s.**
8. **Transfer the vacuum tool to the trough containing 50 ml Wash Buffer (trough 3; Figure 2). Flush the filter probes for 10 s.**

9. **Raise the vacuum tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (Figure 3).**

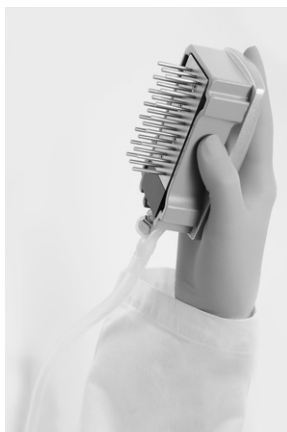


Figure 3. Illustration of the vacuum tool raised to beyond 90° vertical.

10. **While the vacuum 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 vacuum tool to the trough containing high-purity water (trough 4; Figure 2) and agitate it for 10 s.**
13. **Wash the filter probes by lowering the probes into high-purity water (trough 5; Figure 2) and applying vacuum. Flush the probes with 70 ml high-purity water.**
14. **Raise the vacuum tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (Figure 3).**
15. **Close the vacuum switch on the tool (Off), and place the vacuum 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 33.
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 let 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 reagents into the PyroMark Q24 Cartridge, and starting and finishing a run on the PyroMark Q24 System. For detailed description on 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.
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 NRAS 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. Check that the A-peak reduction factor (Analysis Parameters Tab in the Analysis Setup Tab) is set to 0.86 for the NRAS Codon 61 assays.
5. 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*.

6. To generate a report, select **Full Report** from "Reports for AQ runs" in the menu.

The most frequent mutations for each of the three analyzed NRAS codons are found at nucleotide 35 (second base of codon 12), nucleotide 38 (second base of codon 13), and nucleotide 182 (second base of codon 61). Therefore, the standard "Sequence to Analyze" defined in the Analysis Setup addresses mutations at these positions (see Appendix A, page 31). If a sample contains a mutation at nucleotide 34, nucleotide 37, or nucleotide 181, the "Sequence to Analyze" can be changed to also analyze the mutation status at this position, as described in Appendix A. Updated frequencies of mutations in the human NRAS gene in codons 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, 38, or 182 with “Check” or “Failed” quality assessment

We strongly recommend reanalyzing all samples with no mutation detected with the indicated “Sequence to Analyze” in nucleotide 35, 38, or 182, as well as samples which received “Check” or “Failed” quality assessment. “Check” and “Failed” quality assessments may indicate a mutation in a position other than nucleotide 35, 38, or 182, resulting in peak height deviations at reference dispensations. For example, a peak in any of the first 3 dispensations in the codons 12/13 assay shows that a mutation is present at nucleotide 34 of codon 12.

To reanalyze and target mutations at nucleotides 34 and 37, go to “Analysis Setup” and change “Sequence to Analyze” from **GNTGNTGTTGGGAAAAGC** to **NGTNGTGTTGGGAAAAGC**. Click “Apply”, and then click “To All” when the “Apply Analysis Setup” window appears.

To reanalyze and target mutations at nucleotide 181, go to “Analysis Setup” and change “Sequence to Analyze” from **CNAGAAGAGTA** to **VAAGAAGAGTA**.

To reanalyze and target mutations at nucleotide 183, change “Sequence to Analyze” to **CANGAAGAGTA**. Click “Apply”, and then click “To All” when the “Apply Analysis Setup” window appears.

Note: Ensure 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.

Rerunning samples for detection of low-level mutations

It is strongly recommended that a normal sample is included in every run for comparison. Any sample showing a mutation frequency slightly higher than the corresponding position in the normal sample should be re-analyzed. The samples can also be compared with each other to reveal low mutation frequencies.

As a guide, samples that have a suspected low level mutation should be reanalyzed in duplicate together with a normal sample in duplicate. If both duplicates give the same result as the original analysis and are visibly different from the normal control, then the sample can be considered to be positive for the mutation.

Representative results

Representative Pyrogram results are shown in Figures 4–8.

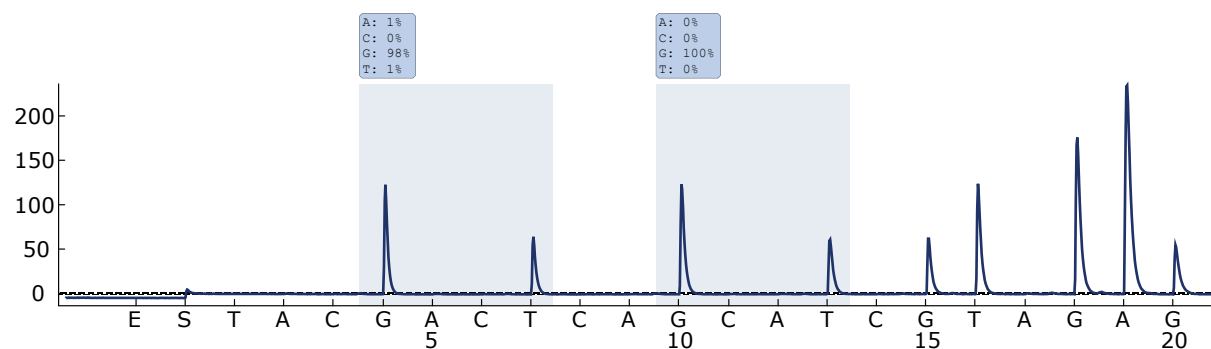


Figure 4. Pyrogram trace obtained after analysis of a sample with a normal genotype in codon 12–13.

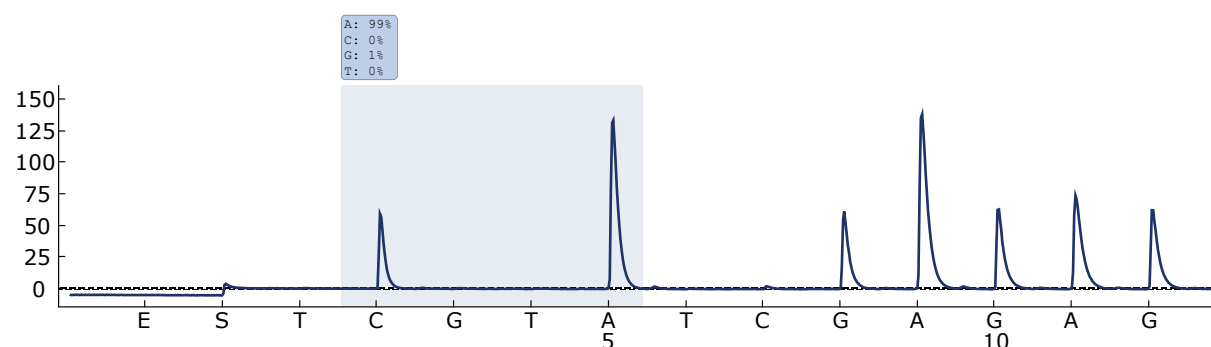


Figure 5. Pyrogram trace obtained after analysis of a sample with a normal genotype in codons 61.

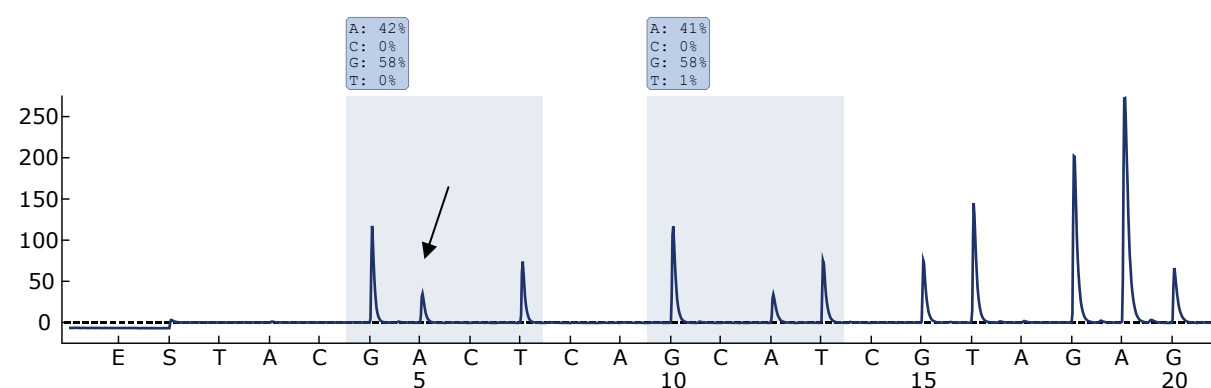


Figure 6. Pyrogram trace obtained after analysis of samples with a GGT → GAT mutation in base 2 of codon 12 (nucleotide 35, indicated with an arrow).

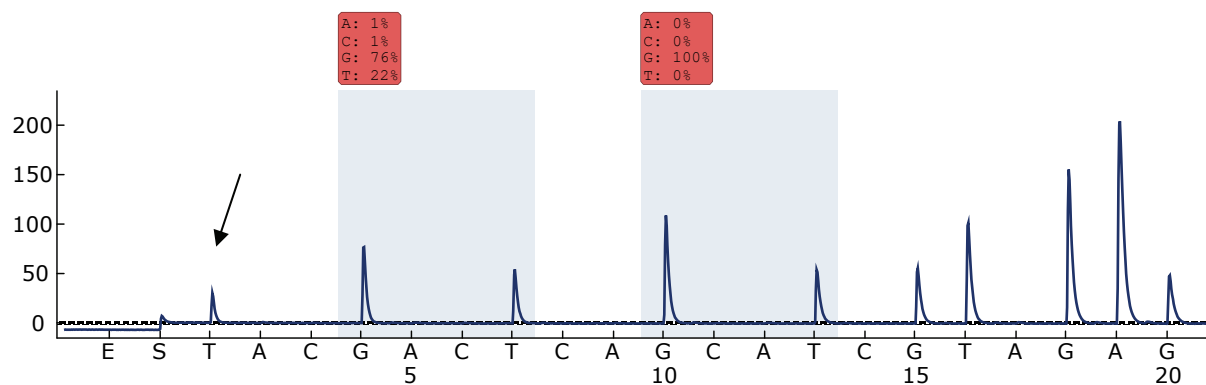


Figure 7. Pyrogram trace obtained after analysis of a sample with a GGT → AGT mutation in base 1 of codon 12 (nucleotide 34, indicated with an arrow) with the “Sequence to Analyze” GNTGNTGTTGGGAAAAGC targeting base 2 in codon 12 (nucleotide 35). A red color indicates that this sequence is unexpected and needs to be checked.

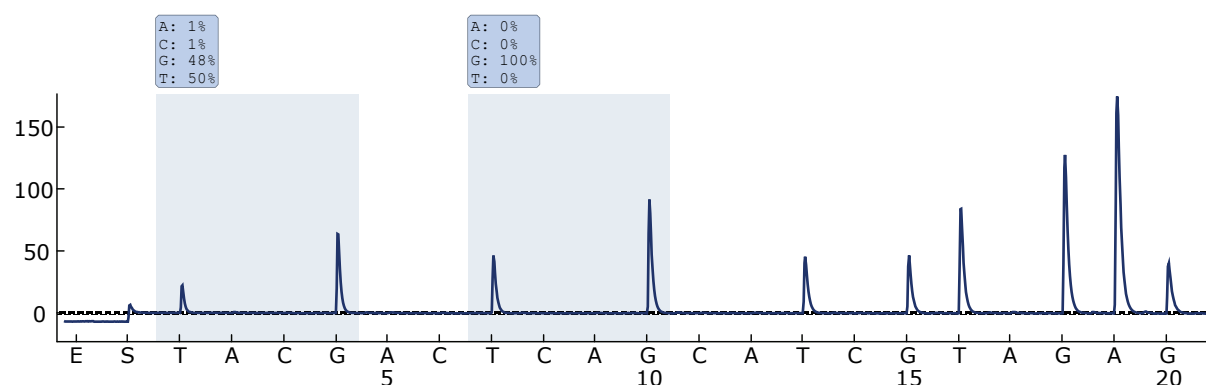


Figure 8. Pyrogram trace and result obtained after reanalysis of the sample in Figure 7. The mutation GGT → AGT was reanalyzed with the “Sequence to Analyze” NGTNGTGTTGGGAAAAGC targeting base 1 in codon 12 (nucleotide 34).

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 "Sequences 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 31), 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 NRAS Pyro Assays

Before running the NRAS Pyro Assay for the first time, the assay file needs to be set up as described below.

Procedure

NRAS codons 12 and 13

A1. Set up the assay for NRAS 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":
GNTGNTGTTGGGAAAAGC

The most frequent mutations in codons 12 and 13 will be detected in nucleotide 35 and 38 (second position) using this "Sequence to Analyze".

The "Sequence to Analyze" can be changed after the run to analyze for mutations at different positions.

To check if mutations are present in nucleotide 34 or 37 (first position), change the "Sequence to Analyze" to the following sequence:

NGTNGTGTTGGGAAAAGC

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

A4. Manually enter the following "Dispensation Order":

TACGACTCAGCATCGTAGAG

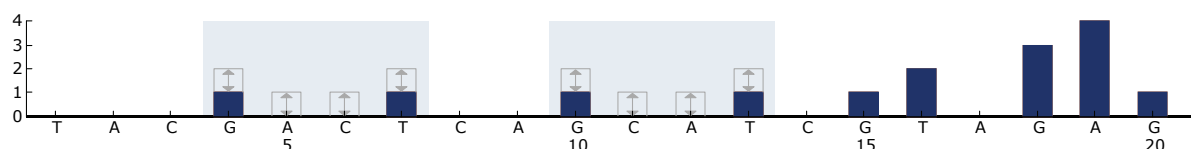


Figure 9. Histogram for codons 12 (nucleotide 35) and 13 (nucleotide 38) with the "Sequence to Analyze" *GNTGNTGTTGGGAAAAGC*.

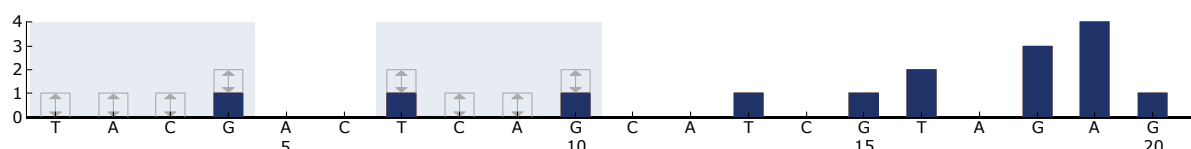



Figure 10. Histogram for codons 12 (nucleotide 34) and 13 (nucleotide 38) with the "Sequence to Analyze" *NGTNGTGTTGGGAAAAGC*.

- 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 “NRAScodons 12+13”.**

NRAS codon 61

- A1. Click  in the toolbar and select “New AQ Assay”.**
- A2. Type the following sequence in “Sequence to Analyze”.**
CNAGAAGAGTA

The most frequent mutation in codon 61 will be detected in nucleotide 182 (second position) with this “Sequence to Analyze”.

The “Sequence to Analyze” can be changed after the run to analyze for mutations in different positions.

To check if mutations are present in nucleotide 181 (first position), change the “Sequence to Analyze” to the following sequence:

VAAGAAGAGTA

To check if mutations are present in nucleotide 183 (third position), change the “Sequence to Analyze” to the following sequence:

CANGAAGAGTA

Note: Ensure 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.

- A3. Manually add the following “Dispensation Order”:**
TCGTATCGAGAG

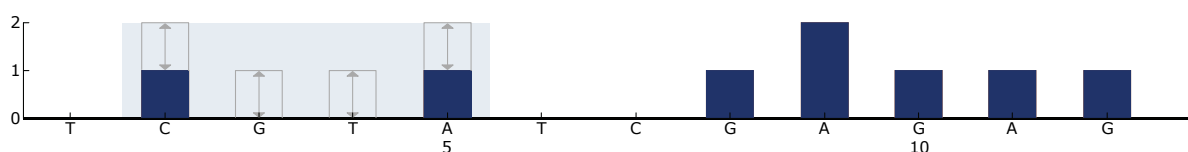


Figure 11. Histogram for codon 61 (nucleotide 182) with the “Sequence to Analyze” CNAGAAGAGTA.

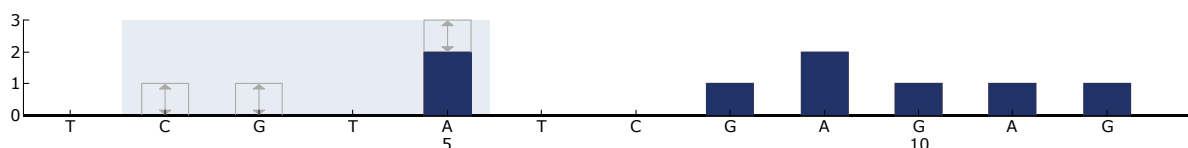


Figure 12. Histogram for codon 61 (nucleotide 181) with the “Sequence to Analyze” VAAGAAGAGTA.

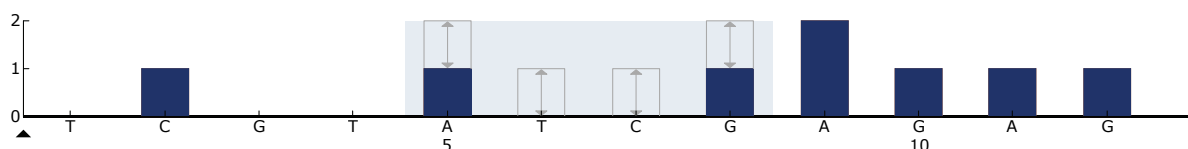





Figure 13. Histogram for codon 61 (nucleotide 183) with the “Sequence to Analyze” CANGAAGAGTA.

- 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 “NRAScodon 61”.**
- A6. Click the “Analysis Parameters” tab, and decrease “A-peak reduction factor:” to 0.86.**
- A7. Click  in the toolbar, and save the assay as “NRAScodon 61”.**

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. 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 Material Safety Data Sheets (MSDSs) 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>
-----------------------------------------------------------------------------------------------------------	-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------

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

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

[‡] COSHH: control of Substances Hazardous to Health (United Kingdom)

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

Important point before starting

- This protocol requires high-purity water.

Procedure

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.

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Ordering Information

Product	Contents	Cat. no.
NRAS 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, dATP α S, dCTP, dGTP, dTTP, and H ₂ O	970530
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 (110V); 9001519 (100V)
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

Ordering Information

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