

NRAS Pyro[®] Handbook

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



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- Nucleic acid and protein assays
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- Automation of sample and assay technologies

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Intended Use

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.

Summary and Explanation

The NRAS Pyro Kit is used for quantitative measurements of mutations in codons 12 and 13 in exon 2 and codon 61 in exon 3 of the human NRAS gene (see Figure 1). The encoded protein is a GTPase.

Exon 2	GTTCTTGCTGGTGTGAAATGACTGAGTACAACTGGTGGTGGTTGGAGCA GGTGGTGTGGGAAAAGCGCACTGACAATCCAGCTAATCCAGAACCACTT TGTAGATGAATATGATCCCACCATAGAG
Exon 3	GATTCTTACAGAAAACAAGTGGTTATAGATGGTGAAACCTGTTTGTGGGA CATACTGGATACAGCTGGACAAAGAAGAGTACAGTGCCATGAGAGACCAAT ACATGAGGACAGGCCGAAGGCTTCCTCTGTGTATTTGCCATCAATAATAGC AAGTCATTTGCGGATATTAACCTCTACAG

Figure 1. Genomic context of the sequenced regions of the human NRAS gene (Ensembl ID ENSG00000213281). Codons 12, 13, and 61 are indicated by boxes.

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

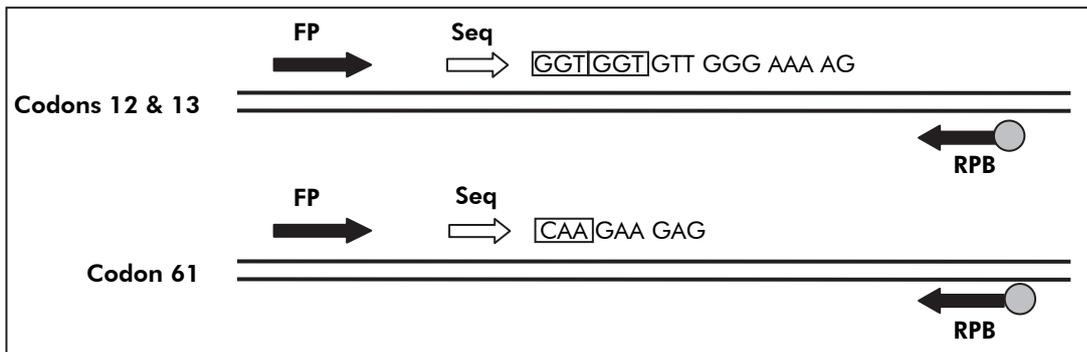


Figure 2. Illustration of the NRAS assay. The sequence indicated is the analyzed sequence for a wild-type sample. **FP:** Forward PCR primers; **RPB:** Reverse PCR primers (B indicates biotinylation); **Seq:** Sequencing primers.

Both assays are sequenced in the forward direction

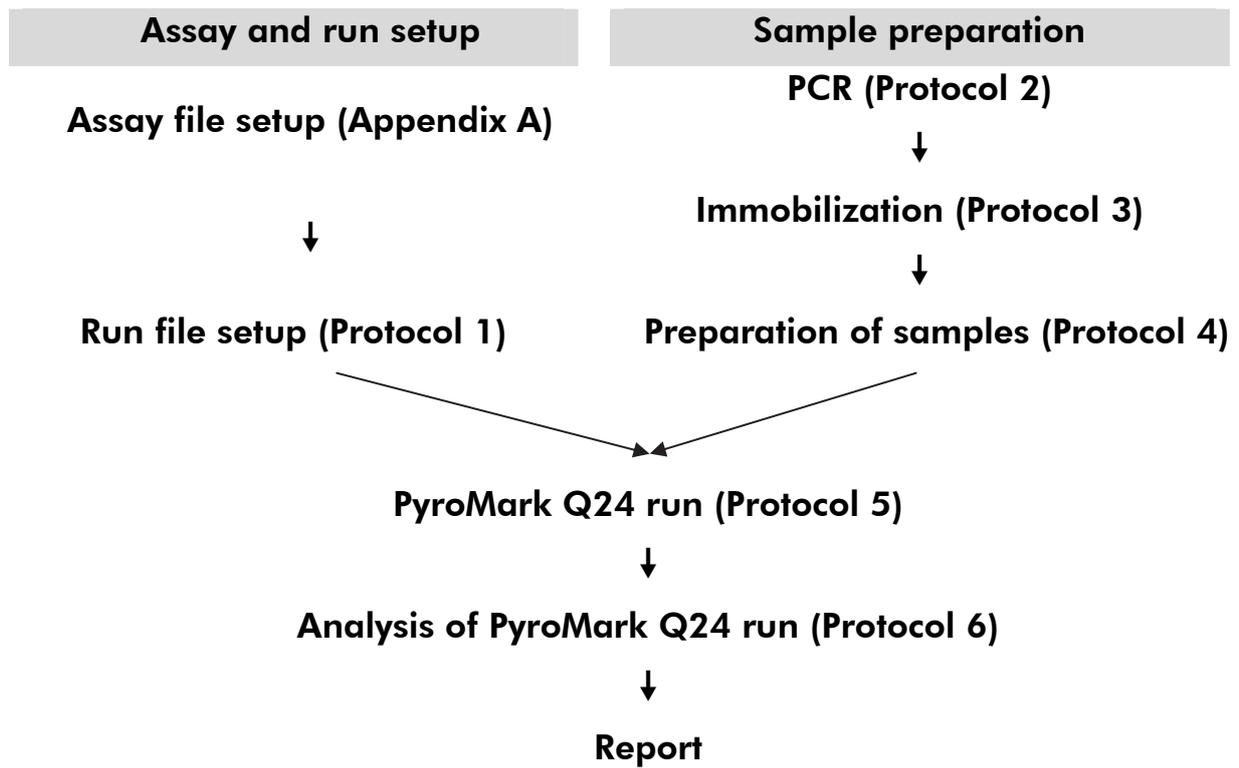
The product consists of a PCR primer mix and a sequencing primer for each assay. The primers are delivered in solution. Each vial contains 24 μ l of each primer or primer mix.

Principle of the Procedure

The workflow below 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 assay setup files 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 27, and “Appendix A: Setting Up NRAS Pyro Assays”, page 35).

Note: The workflow has been slightly modified compared to the previous revision of the *NRAS Pyro Handbook* (September 2010). See “Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads”, page 19 and “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 21.

Workflow of NRAS Pyro procedure



Controls

Unmethylated Control DNA is included in the kit as a positive control for PCR and sequencing reactions. This control DNA has a wild-type genotype in the regions sequenced using this kit and is required for adequate result interpretation and identification of low-level mutations (see “Protocol 6: Analysis of a PyroMark Q24 run”, page 27). Include a sample with Unmethylated Control DNA for each assay in every Pyrosequencing® run.

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

Materials Provided

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.

Materials Required but Not Provided

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

- DNA isolation kit (see "DNA isolation", page 13)
- 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 (see Table 1, below)
- Heating block capable of attaining 80°C
- 24-well PCR plates (see Table 2, page 11)
- Adhesive foil
- High-purity water (Milli-Q® 18.2 MΩ x cm or equivalent)

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

- Ethanol (70%)*

Recommended plate mixers

The orbital plate mixers in Table 1 are recommended for use with the NRAS Pyro Kit.

Table 1. Plate mixers recommended for use with the NRAS Pyro Kit

Manufacturer	Product	Catalog number
Eppendorf	Thermomixer comfort (Basic device)	5355 000.011
	Thermoblock for MTP	5363 000.012
	Adapter plate for 96 x 0.2 ml PCR tubes to insert in blocks for microtiter plates	5363 007.009
H+P Labortechnik GmbH	Variomag® Teleshake	51410 (115 V = 51410 U)
	Variomag Monoshake	51110 (115 V = 51110 U)

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

Recommended 24-well plates

The 24-well plates in Table 2 are recommended for use with the NRAS Pyro Kit.

Table 2. 24-well plates recommended for use with the NRAS Pyro Kit

Manufacturer	Product	Catalog number
ABgene (Thermo Scientific)	Thermo-Fast PCR Plate	AB-0624
Axygen	24 Well PCR Microplate	PCR-24-C
4titude	FrameStar® Break-a-way 96 wells, clear tubes	4ti-1000
Kisker	Quali – PCR Plates without frame	G030

Warnings and Precautions

For molecular biology applications.

Safety information

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

General precautions

The user should always pay attention to the following:

- The workflow has been slightly modified compared to the previous revision of the *NRAS Pyro Handbook* (September 2010). See “Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads”, page 19, and “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 21.

- The components of this product are sufficient to perform 24 reactions in up to 5 independent runs.
- Use sterile pipet tips (with filters for PCR setup).
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.
- Failed results are not a basis for judgment of mutational status.

Reagent Storage and Handling

The NRAS Pyro Kit is shipped in 2 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 10 days at 2–8°C. Reconstituted enzyme and substrate mixtures can be frozen and stored in their vials at –15 to –25°C. Frozen reagents should not be subjected to more than 6 freeze–thaw cycles.

Note: Nucleotides should not be frozen.

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

Specimen Handling and Storage

All samples must be treated as potentially infectious material.

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

Procedure

DNA isolation

The kits from QIAGEN shown in Table 3 are recommended for DNA purification from the indicated human sample types for use with the NRAS Pyro Kit. Carry out DNA purification according to the instructions in the kit handbooks.

Table 3. 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 DNA Blood Mini Kit (50)	51104

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

Protocol 1: Run setup for the PyroMark Q24 system

Things to do before starting

- Create an Assay Setup as described in Appendix A. This needs to be done only once, before running the NRAS Pyro assay for the first time (see “Appendix A: Setting Up NRAS Pyro Assays”, page 35).

Procedure

1. Click  in the toolbar.

A new run file is created.

2. Enter the run parameters (see “Run parameters”, page 15).

3. Set up the plate by adding assays for both codons 12/13 and codon 61 to wells corresponding to the samples to analyze.

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

Note: Include a sample with Unmethylated Control DNA for each assay in every Pyrosequencing run (“Controls”, page 7).

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 19).

To run the plate on the PyroMark Q24 system, see “Protocol 5: Running the PyroMark Q24”, page 25.

Run parameters

Run name:	The name of the run is given when the file is saved. Renaming the file also changes the name of the run.
Instrument method:	Select the instrument method according to the cartridge that will be used for the run; see the instructions supplied with the products.
Plate ID:	Optional: Enter ID of the PyroMark Q24 Plate.
Bar code:	Optional: Enter a bar code number for the plate or, if you have a bar code reader connected to your computer, place the mouse cursor in the "Barcode" text box (by clicking the box) and scan the bar code.
Kit and reagent ID:	Optional: Enter the lot number for the NRAS Pyro Kit box 1 and box 2 to be used. The lot number can be found on the product label. Note: We recommend entering both lot numbers so that any unexpected problems with the NRAS Pyro Kit can be traced.
Run note:	Optional: Enter a note about the contents or purpose of the run.

Add assay files

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

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

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

Enter sample IDs and notes

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

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

Protocol 2: PCR using the 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 PCR Master Mix requires an activation step of **15 minutes at 95°C**.
- Set up all reaction mixtures in an area separate from that used for DNA purification, adding template to the PCR, PCR product analysis, or preparation of samples prior to Pyrosequencing analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

Things to do before starting

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

Procedure

1. Thaw all necessary components (see Table 4, page 17).

Mix well before use.

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

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

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

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

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

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 5), and mix thoroughly.

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

Note: Include a sample with Unmethylated Control DNA for each assay in every Pyrosequencing run (see “Controls”, page 7).

Table 5. Preparation of PCR

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

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

Table 6. Optimized cycling protocol

			Comments
Initial activation step:	15 minutes	95°C	HotStarTaq DNA polymerase is activated by this heating step.
3-step cycling:			
Denaturation	20 seconds	95°C	
Annealing	30 seconds	53°C	
Extension	20 seconds	72°C	
Number of cycles	42		
Final extension:	5 minutes	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 19.**

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

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

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

Important point before starting

- Note that the workflow has been slightly modified compared to the previous revision of the *NRAS Pyro Handbook* (September 2010, step 2).

Things to do before starting

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

Note: The 1x PyroMark Wash Buffer working solution is stable at 2–8°C until the marked expiration date.

- Prepare the PyroMark Q24 Vacuum Workstation for sample preparation as described in the *PyroMark Q24 User Manual*.

Procedure

- 1. Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.**
- 2. Prepare a master mix for DNA immobilization according to Table 7, page 20.**

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

Table 7. Master mix for DNA immobilization

Component	Volume/sample (µl)
PyroMark Binding Buffer	40
Water (H ₂ O, supplied)	29
Streptavidin Sepharose High Performance	1
Total volume	70

Note: This protocol applies to Streptavidin Sepharose High Performance with lot number 10057037 or higher. When using Streptavidin Sepharose High Performance beads with a lot number lower than 10057037, the volume of beads per sample used must be increased to 2 µl, while reducing the volume of water appropriately.

- 3. Add 70 µl of the master mix to wells of a 24-well PCR plate as predefined in the run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 14).**

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

- 4. Add 10 µl of biotinylated PCR product from Protocol 2 to each well containing master mix as predefined in the run setup (see “Protocol 2: PCR using the reagents supplied with the NRAS Pyro Kit”, page 16).**

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

- 5. Seal the PCR plate using adhesive foil.**

Ensure that no leakage is possible between the wells.

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

During this step, proceed immediately with “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 21.

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 14), depending on the region of analysis (codons 12 and 13, or codon 61).
- Note that the workflow has been slightly modified compared to the previous revision of the *NRAS Pyro Handbook* (September 2010, step 18). Do not shorten the time for cooling down the samples after heating to 80°C.
- Perform the function test for the filter probes as described in the *PyroMark Q24 User Manual* on a regular basis and exchange filter probes when indicated.

Procedure

1. **Dilute a sufficient amount of each sequencing primer, Seq Primer NRAS 12/13, and Seq Primer NRAS 61, in PyroMark Annealing Buffer as shown in Table 8.**

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

Do not dilute and store more sequencing primer.

Table 8. Example of dilution of the sequencing primers

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

- 2. Add 25 μl of diluted sequencing primer to each well of the PyroMark Q24 Plate according to the run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 14).**

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

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

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



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

- 5. Apply vacuum to the tool by opening the vacuum switch.**

- 6. Slowly lower the filter probes of the vacuum tool into the PCR plate to capture the beads containing immobilized template. Hold the probes in place for 15 seconds. Take care when picking up the vacuum tool.**

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

Inspect the PCR plate for a complete take-up of all samples by the vacuum tool.

- 7. Transfer the vacuum tool to the trough containing 40 ml 70% ethanol (trough 1; Figure 3). Flush the filter probes for 5 seconds.**
- 8. Transfer the vacuum tool to the trough containing 40 ml Denaturation Solution (trough 2; Figure 3). Flush the filter probes for 5 seconds.**
- 9. Transfer the vacuum tool to the trough containing 50 ml Wash Buffer (trough 3; Figure 3). Flush the filter probes for 10 seconds.**
- 10. Raise the vacuum tool up and back, beyond 90° vertical, for 5 seconds to drain liquid from the filter probes (Figure 4).**



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

- 11. While the vacuum tool is held over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).**
- 12. Release the beads into the PyroMark Q24 Plate by lowering the filter probes into the diluted sequencing primer and moving the vacuum tool gently from side to side.**

Take care not to damage the surface of the PyroMark Q24 Plate by scratching it with the filter probes.
- 13. Transfer the vacuum tool to the trough containing high-purity water (trough 4; Figure 3) and agitate it for 10 seconds.**
- 14. Wash the filter probes by lowering the probes into high-purity water (trough 5; Figure 3) and applying vacuum. Flush the probes with 70 ml high-purity water.**

- 15. Raise the vacuum tool up and back, beyond 90° vertical, for 5 seconds to drain liquid from the filter probes (Figure 4).**
- 16. Close the vacuum switch on the tool (Off), and place the vacuum tool in the Parking (P) position.**
- 17. Turn off the vacuum pump.**

At the end of a workday, liquid waste and remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage. See “Appendix B: Emptying the Waste Container and Troughs”, page 38.
- 18. Heat the PyroMark Q24 Plate with the samples at 80°C for 2 minutes using the pre-warmed PyroMark Q24 plate holder.**
- 19. Remove the PyroMark Q24 Plate from the hot plate holder, and place it on a second PyroMark Q24 plate holder that was kept at room temperature (15–25°C) to let the samples cool to room temperature for 10–15 minutes.**
- 20. Proceed with “Protocol 5: Running the PyroMark Q24”, page 25.**

Protocol 5: Running the PyroMark Q24

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

Important points before starting

- The Pre Run information report, found in the “Tools” menu at run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 14), provides information about the volume of nucleotides, enzyme, and substrate buffer needed for a specific run.
- Use disposable tips without hydrophobic filters for loading the cartridge to permit correct function of the cartridge.

Procedure

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

Do not vortex!

To ensure that the mixture is fully dissolved, leave it at room temperature (15–25°C) for 5–10 minutes. Make sure that the solution is not turbid before filling the PyroMark Q24 Cartridge. If the reagents are not to be used immediately, place the reagent vials on ice or in a refrigerator.

- 3. Allow the reagents and the PyroMark Q24 Cartridge to reach ambient temperature (20–25°C).**
- 4. Place the PyroMark Q24 Cartridge with the label facing you.**
- 5. Load the PyroMark Q24 Cartridge with the appropriate volumes of nucleotides, enzyme, and substrate mixes according to Figure 5.**

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

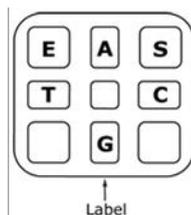


Figure 5. Illustration of the PyroMark Q24 Cartridge as seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (**E**), substrate mixture (**S**), and nucleotides (**A**, **T**, **C**, **G**) according to the volume information given in the Pre Run information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

6. **Open the cartridge gate and insert the filled reagent cartridge with the label facing out. Push the cartridge in fully and then push it down.**
7. **Ensure the line is visible in front of the cartridge and close the gate.**
8. **Open the plate-holding frame and place the plate on the heating block.**
9. **Close the plate-holding frame and the instrument lid.**
10. **Insert the USB stick (containing the run file) into the USB port at the front of the instrument.**
Do not remove the USB stick before the run is finished.
11. **Select “Run” in the main menu (using the ▲ and ▼ screen buttons) and press “OK”.**
12. **Select the run file using the ▲ and ▼ screen buttons.**
To view the contents of a folder, select the folder and press “Select”. To go back to the previous view, press “Back”.
13. **When the run file is selected, press “Select” to start the run.**
14. **When the run is finished and the instrument confirms that the run file has been saved to the USB stick, press “Close”.**
15. **Remove the USB stick.**
16. **Open the instrument lid.**
17. **Open the cartridge gate and remove the reagent cartridge by lifting it up and pulling it out.**
18. **Close the gate.**
19. **Open the plate-holding frame and remove the plate from the heating block.**
20. **Close the plate-holding frame and the instrument lid.**
21. **Discard the plate and clean the cartridge, as per the instructions in the product sheet supplied with the cartridge.**
22. **Analyze the run according to “Protocol 6: Analysis of a PyroMark Q24 run”, page 27.**

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 "AQ Full Report" or "AQ Analysis Results" in the "Reports" menu.

Note: For reliable results, we recommend single peak heights above 30 RLU. Set 30 RLU as the "required peak height for passed quality" in assay setup (see "Appendix A: Setting Up NRAS Pyro Assays" and the *PyroMark Q24 User Manual*).

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

Note: The Pyrogram should always be compared to the histogram, which can be displayed by right-clicking in the Pyrogram window. The measured peaks should match the height of the histogram bars.

Reanalysis of samples with no mutation detected in nucleotide 35, 38, or 182 or with "Check" or "Failed" quality assessment

The most frequent mutations for each of the 3 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 Assay Setup address mutations at these positions (Table 9, page 29 and "Appendix A: Setting Up NRAS Pyro Assays", page 35).

We strongly recommend reanalyzing all samples with no mutation detected with the standard "Sequence to Analyze", as well as samples which received "Check" or "Failed" quality assessment, or show peaks that do not match the height of the histogram bars. "Check" and "Failed" quality assessments may indicate a mutation in a position other than nucleotide 35, 38, or 182, resulting in peak height deviations. 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, 37, 181, and 183, go to "Analysis Setup" and change the standard "Sequence to Analyze" to one of the additional "Sequence to Analyze" listed in Table 9, page 29. Click "Apply", and then click "To All" when the "Apply Analysis Setup" window appears (see "Appendix A: Setting Up NRAS Pyro Assays", page 35).

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/.

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

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

Note: In case the measured peaks do not match the height of the histogram bars and cannot be explained by rare or unexpected mutations, we recommend rerunning the sample.

Table 9. Common mutations detected by the NRAS Pyro Kit with respective "Sequence to Analyze"

Nucleic acid substitution	Amino acid substitution	"Sequence to Analyze"	COSMIC ID* (V47)
Codon 12 (GGT)			
AGT	G12S	NGTNGTGTTGGGAAAAGC	563
TGT	G12C	NGTNGTGTTGGGAAAAGC	562
CGT	G12R	NGTNGTGTTGGGAAAAGC	561
GAT	G12D	GNTGNTGTTGGGAAAAGC[†]	564
GTT	G12V	GNTGNTGTTGGGAAAAGC[†]	566
GCT	G12A	GNTGNTGTTGGGAAAAGC[†]	565
Codon 13 (GGT)			
AGT	G13S	NGTNGTGTTGGGAAAAGC	571
TGT	G13C	NGTNGTGTTGGGAAAAGC	570
CGT	G13R	NGTNGTGTTGGGAAAAGC	569
GAT	G13D	GNTGNTGTTGGGAAAAGC[†]	573
GTT	G13V	GNTGNTGTTGGGAAAAGC[†]	574
GCT	G13A	GNTGNTGTTGGGAAAAGC[†]	575
Codon 61 (CAA)			
AAA	Q61K	VAAGAAGAGTA	580
CGA	Q61R	CNAGAAGAGTA[†]	584
CTA	Q61L	CNAGAAGAGTA[†]	583
CAT	Q61H	CANGAAGAGTA	585
CAC	Q61H	CANGAAGAGTA	586
CAG	Q61Q	CANGAAGAGTA	587

* Sanger Institute at www.sanger.ac.uk/genetics/CGP/cosmic/.

[†] Standard "Sequence to Analyze".

Detection of low-level mutations

We strongly recommend that the Unmethylated Control DNA is included in every run for comparison and as a control for background levels.

Samples showing a mutation frequency only slightly higher than the corresponding position in the control sample should be rerun in duplicate together with a sample of Unmethylated Control DNA to confirm the mutational status.

Representative results

Representative Pyrogram results are shown in Figures 6–10.

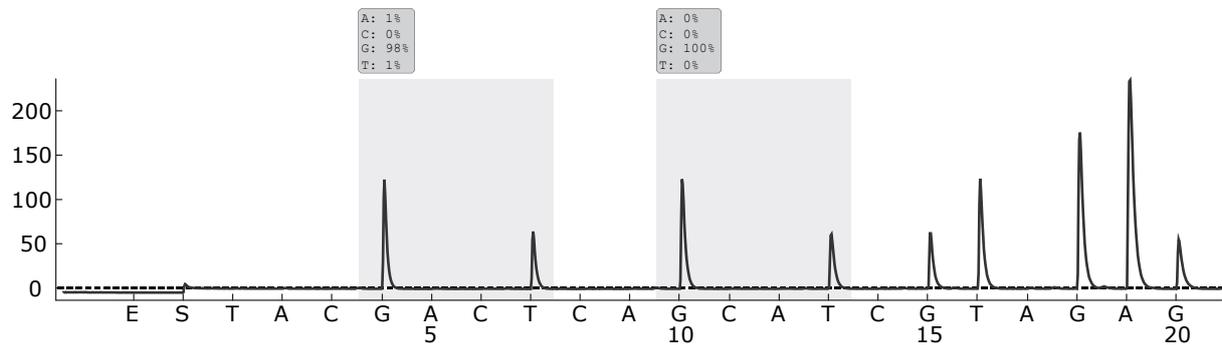


Figure 6. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codon 12–13.

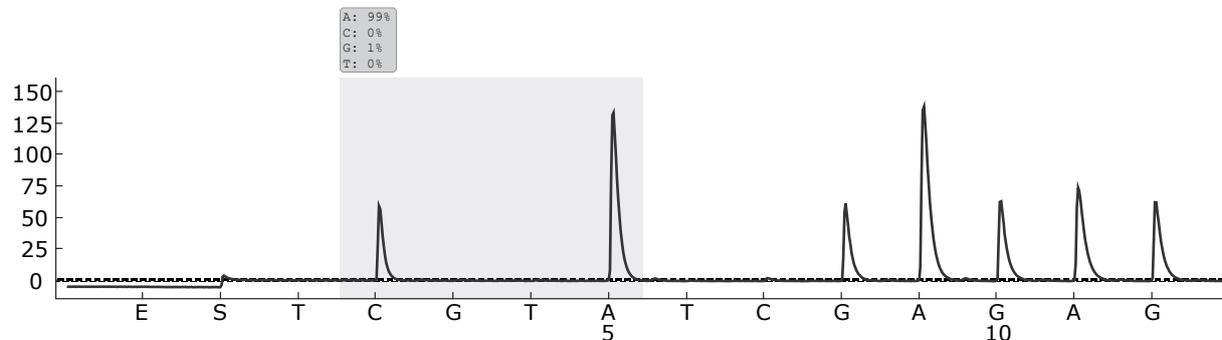


Figure 7. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codons 61.

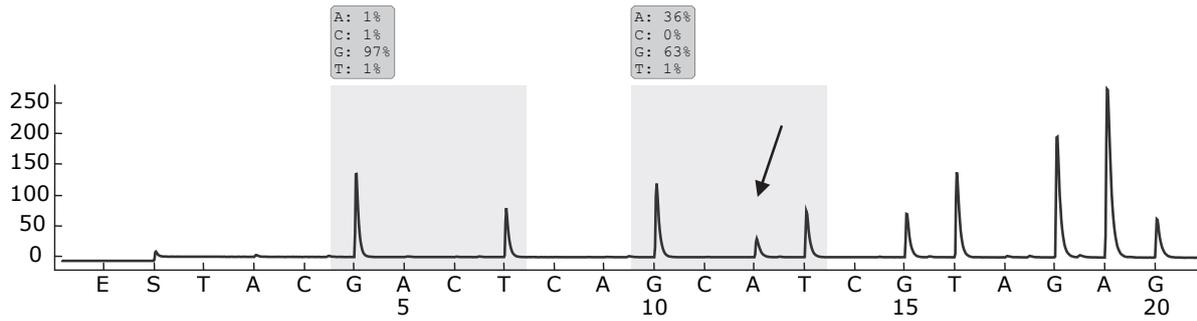


Figure 8. Pyrogram trace obtained after analysis of a sample with a GGT → GAT mutation in base 2 of codon 13 (nucleotide 38, indicated with an arrow) with the “Sequence to Analyze” GNTGNTGTTGGGAAAAGC.

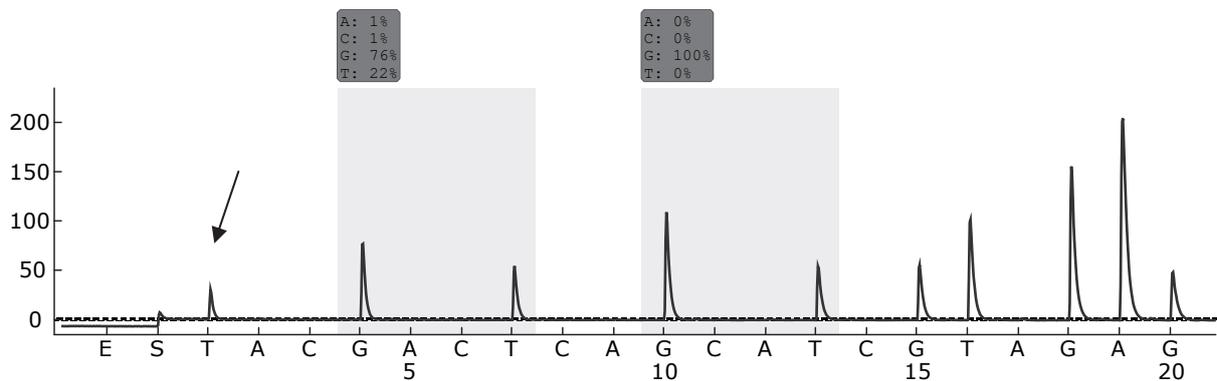


Figure 9. Pyrogram trace obtained after analysis of a sample with a GGT → AGT mutation in base 1 of codon 12 (nucleotide 34, indicated with an arrow) with the “Sequence to Analyze” GNTGNTGTTGGGAAAAGC targeting base 2 in codon 12 (nucleotide 35). A red color indicates that this sequence is unexpected and needs to be checked.

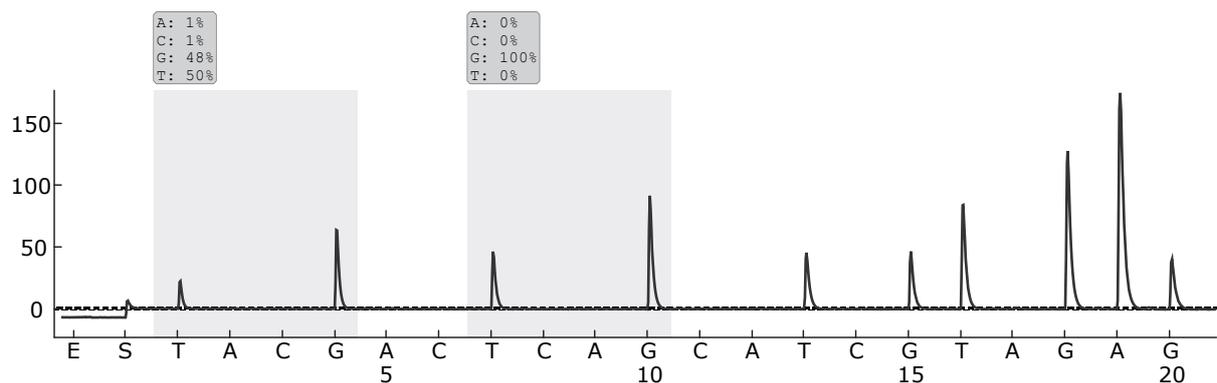


Figure 10. Pyrogram trace and result obtained after reanalysis of the sample in Figure 9. The mutation GGT → AGT was reanalyzed with the “Sequence to Analyze” NGTNGTGTGGGAAAAGC targeting base 1 in codon 12 (nucleotide 34).

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. 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, controls, and amplicons separately from PCR reagents. |

Poor or unexpected sequence

- | | |
|-------------------------|---|
| Low-quality genomic DNA | Low-quality genomic DNA can cause the PCR to fail. Analyze PCR samples using an electrophoretic technique (for example, the QIAxcel [®] Advanced system or agarose-gel electrophoresis). |
|-------------------------|---|

“Check” or “failed” result

- a) Low peak height Handling errors in PCR setup or sample preparation prior to Pyrosequencing can result in low peaks.
- It is important that the samples are completely taken up by the vacuum tool. Take care that the vacuum tool is lowered slowly into the samples and that the geometry of the PCR plate or strips used for immobilization allows complete take-up of the samples.
- Perform the function test for filter probes as described in the *PyroMark Q24 User Manual* on a regular basis, and exchange filter probes when indicated.
- In case of a “Check” warning, carefully compare the Pyrogram to the histogram, which can be displayed by right-clicking in the Pyrogram window. If the measured peaks match the height of the histogram bars, the result is valid. Otherwise, we recommend rerunning the sample.
- b) Mutation not defined in the “Sequence to Analyze” Adjust the “Sequence to Analyze” in the assay setup (see “Appendix A: Setting Up NRAS Pyro Assays”, page 35), and reanalyze the run.
- c) Unexpected rare mutation A “Check” or “Failed” quality assessment can be caused by an unexpected pattern of peaks. This might indicate an unexpected mutation, which is not analyzed by the provided “Sequence to Analyze”. These samples should be analyzed using the alternative “Sequences to Analyze” considering unexpected mutations.
- d) High peak height deviation warning for a dispensation The Pyrogram should be carefully compared to the histogram, which can be displayed by right-clicking in the Pyrogram window. In case the measured peaks do not match the height of the histogram bars and cannot be explained by rare mutations, we recommend rerunning the sample.

High background

- | | |
|---|---|
| a) Incorrect storage of nucleotides | Store nucleotides at 2–8°C. Storage at –15 to –25°C can cause an increase in the background. |
| b) Short cooling time of samples prior to Pyrosequencing analysis | Keep the samples on a PyroMark Q24 plate holder at room temperature for 10–15 minutes. Do not shorten the cooling time. |
| c) Contamination of cartridge | Carefully clean the cartridge as described in the product sheet. Store the cartridge protected from light and dust. |

No signals in positive control (Unmethylated Control DNA)

- | | |
|---|---|
| a) Insufficient enzyme or substrate mix for all wells | Make sure to fill the PyroMark Q24 Cartridge according to the “Pre Run Information” in the “Tools” menu. |
| b) Reagents incorrectly stored or diluted | Prepare the reagents according to the instructions in “Protocol 5: Running the PyroMark Q24”, page 25. |
| c) PCR or sample preparation failure | Handling errors in PCR setup, programming of the PCR cycler, or sample preparation prior to Pyrosequencing can result in no signals. Perform the function test for filter probes as described in the <i>PyroMark Q24 User Manual</i> , and exchange filter probes when indicated. Repeat the PCR and Pyrosequencing analysis. |

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.

Contact Information

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

Appendix A: Setting Up NRAS Pyro Assays

Before running the NRAS Pyro Assays for the first time, the assay files must be set up once using the PyroMark Q24 Software, as described below.

Procedure

NRAS codons 12 and 13

A1. Click  in the toolbar and select “New AQ Assay”.

A2. 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 (see “Protocol 6: Analysis of a PyroMark Q24 run”, page 27).

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.

A3. Manually enter the following “Dispensation Order”:
TACGACTCAGCATCGTAGAG

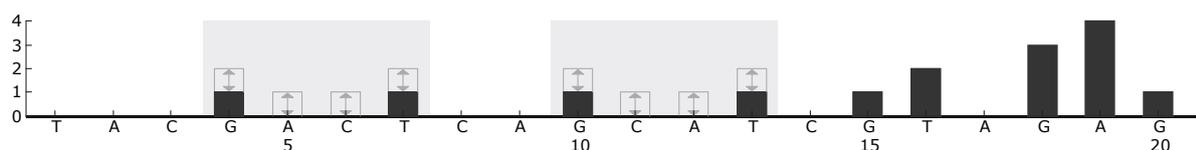


Figure 11. Histogram for codons 12 (nucleotide 35) and 13 (nucleotide 38) with the “Sequence to Analyze” GNTGNTGTTGGGAAAAGC.

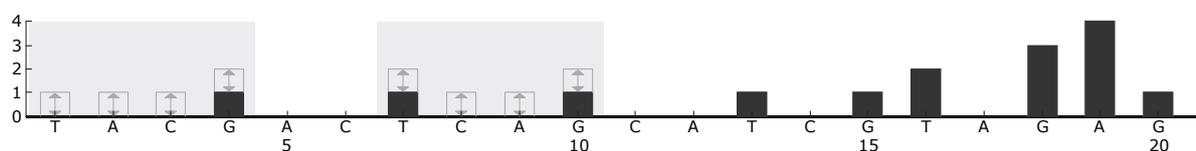


Figure 12. Histogram for codons 12 (nucleotide 34) and 13 (nucleotide 37) with the “Sequence to Analyze” NGTNGTGTTGGGAAAAGC.

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 "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

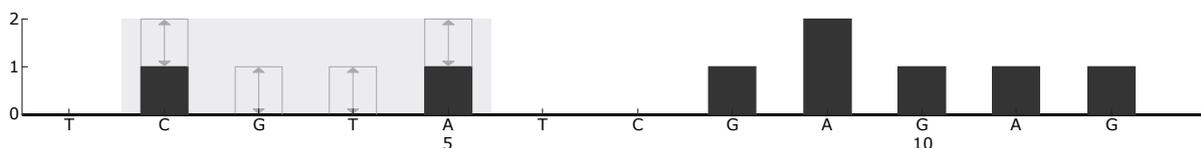


Figure 13. Histogram for codon 61 (nucleotide 182) with the "Sequence to Analyze" CNAGAAGAGTA.

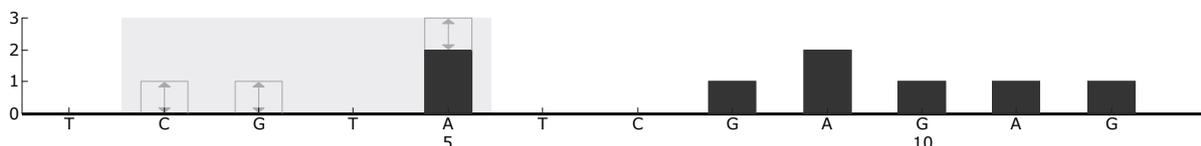


Figure 14. Histogram for codon 61 (nucleotide 181) with the "Sequence to Analyze" VAAGAAGAGTA.

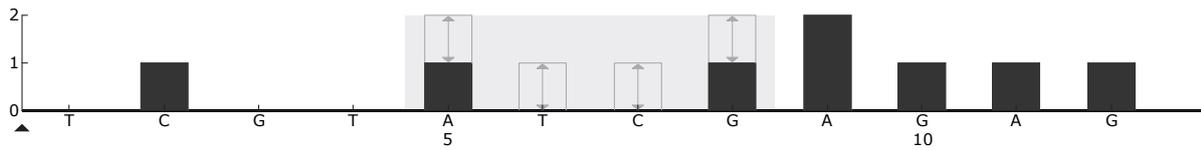


Figure 15. Histogram for codon 61 (nucleotide 183) with the “Sequence to Analyze” CANGAAGAGTA.

- A4. Click the “Analysis Parameters” tab, and increase “Peak Height Threshold - Required peak height for Passed quality:” to 30.**
- A5. Click the “Analysis Parameters” tab, and decrease “A-peak reduction factor:” to 0.86.**
- A6. 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 Safety Data Sheets (SDSs) or OSHA,* ACGIH,[†] or COSHH[‡] documents.</p> <p>Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.</p>
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* OSHA: Occupational Safety and Health Administration (United States of America)

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

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

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

Important point before starting

- This protocol requires high-purity water.

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.

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 (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

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 DNA Blood Mini Kit (50)	For 50 preps: QIAamp Mini Spin Columns, QIAGEN Protease, Reagents, Buffers, Collection Tubes (2 ml)	51104
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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Notes

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