

EGFR Pyro[®] Handbook

For quantitative measurement of mutations in codons 719, 768, 790, 858, and 861, as well as deletions and complex mutations in exon 19 of the human EGFR gene



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Kit Contents

EGFR Pyro Kit (box 1/2)

EGFR Pyro Kit	(24)
Catalog no.	970480
Number of reactions	24
Seq Primer EGFR 719	24 µl
Seq Primer EGFR Ex 19 Del	24 µl
Seq Primer EGFR 768	24 µl
Seq Primer EGFR 790	24 µl
Seq Primer EGFR 858–861	24 µl
PCR Primer EGFR 719	24 µl
PCR Primer EGFR Ex19 Del	24 µl
PCR Primer EGFR 768+790	24 µl
PCR Primer EGFR 858–861	24 µl
PyroMark® PCR Master Mix, 2x	2 x 850 µl
CoralLoad® Concentrate, 10x	1.2 ml
H ₂ O	5 x 1.9 ml
Unmethylated Control DNA, 10 ng/µl	100 µl

Pyro Buffers and Reagents (box 2/2)

Pyro Buffers and Reagents	
PyroMark Binding Buffer	2 x 10 ml
PyroMark Annealing Buffer	2 x 10 ml
PyroMark Denaturation Solution*	2 x 250 ml
PyroMark Wash Buffer, 10x	2 x 25 ml
Enzyme Mixture	2 vials
Substrate Mixture	2 vials
dATP α S	2 x 1180 μ l
dCTP	2 x 1180 μ l
dGTP	2 x 1180 μ l
dTTP	2 x 1180 μ l
Handbook	1

* Contains sodium hydroxide.

Shipping and Storage

The EGFR Pyro Kit is shipped in two boxes. The EGFR 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 EGFR Pyro Kit is stable until the kit expiration date when stored under these conditions.

Product Use Limitations

The EGFR 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 EGFR 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 EGFR 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 EGFR Pyro Kit enables quantitative measurement of mutations in codons 719, 768, 790, 858, and 861, as well as deletions and complex mutations in exon 19 of the human EGFR gene. The kit consists of 4 PCR assays: one for detecting mutations in codon 719 (exon 18), one for detecting mutations in codons 768 and 790 (exon 20), one for detecting mutations in codons 858 to 861 (exon 21), as well as one for detecting deletions and complex mutations in exon 19. The 4 regions are amplified separately by PCR and sequenced through the defined region. The amplicon covering codons 768 and 790 is divided into 2 sequencing reactions. Sequences surrounding the defined positions serve as normalization and reference peaks for quantification and quality assessment of the analysis.

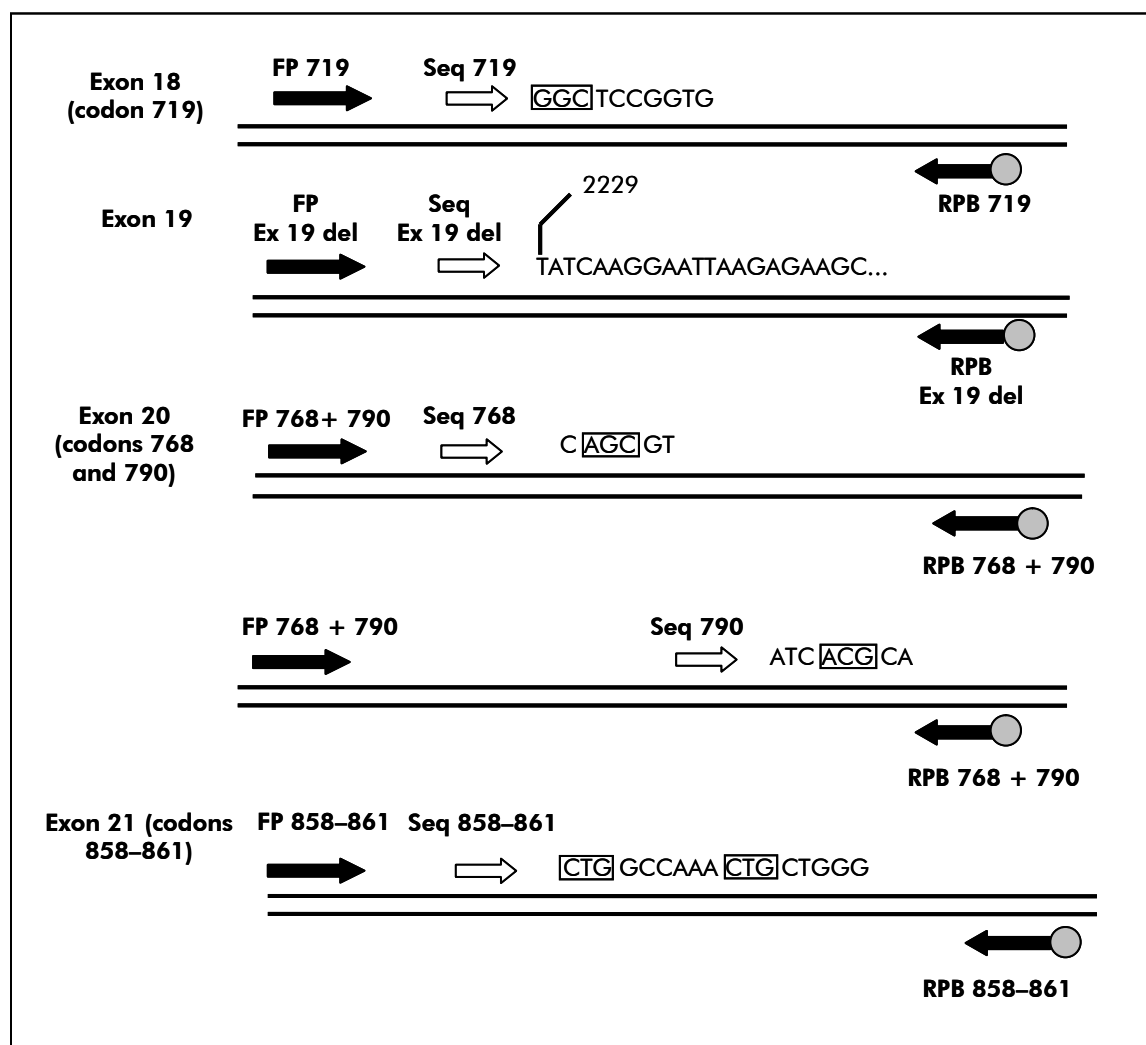


Figure 1. Illustration of the EGFR assay. The sequence indicated is the analyzed sequence for a normal sample. **FP:** Forward PCR primers; **RPB:** Reverse PCR primers (B indicates biotinylation); **Seq:** Sequencing primers.

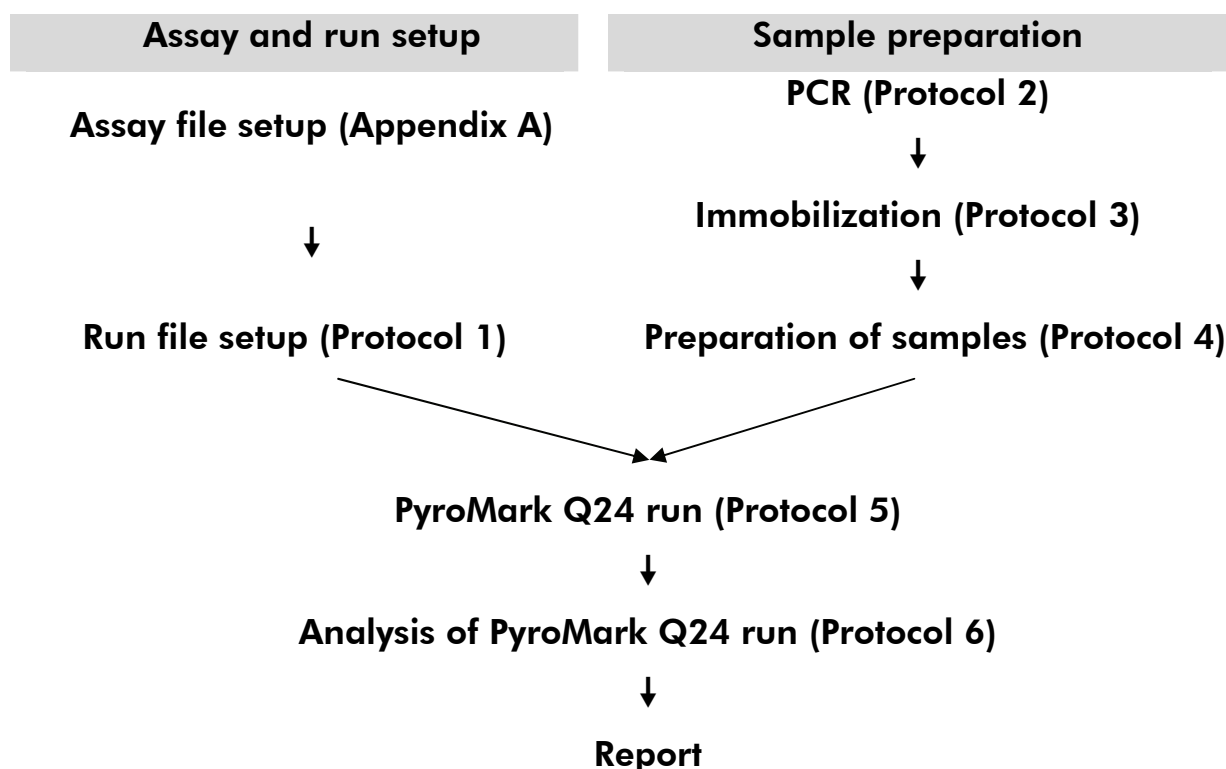
All mutations are sequenced in the forward direction.

The kit consists of a PCR primer mix and sequencing primer(s) 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 below illustrates the assay procedure. After PCR using primers targeting exons 18, 19, 20, and 21, the amplicons are immobilized on Streptavidin Sepharose[®] High Performance beads. Single-stranded DNA is prepared, and the corresponding sequencing primers anneal to the DNA. The samples are then analyzed on the PyroMark Q24 System using a run setup file and a run file. It is recommended to use the EGFR Plug-in Report to analyze the run. However, the run can also be analyzed using the analysis tool integral to the PyroMark Q24 System. The "Sequence to Analyze" can be then adjusted for detection of different deletions in exon 19 and of rare mutations in the other exons after the run (see "Protocol 6: Analysis of a PyroMark Q24 Run", page 26, and Appendix A, page 35).

Workflow of EGFR 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:

- Strict compliance with the user manual is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended, and will result in a loss of performance.
- Use sterile pipet tips (with filters for PCR setup).
- Store and extract positive materials (specimens, positive controls, and amplicons) separately from all other reagents, and add them to the reaction mix in a spatially separated facility.
- Thaw all components thoroughly at room temperature (15–25°C) before starting an assay.
- When thawed, mix the components (by pipetting repeatedly up and down or by pulse vortexing) and centrifuge briefly.

Sample material

All samples must be treated as potentially infectious material.

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

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

DNA isolation

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

Things to do before starting

- If the EGFR Plug-in Report for analysis has not been installed, create an Assay Setup as described in Appendix A. This must only be done once, before running the EGFR assays for the first time (see Appendix A, page 35).

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 the 5 different sequencing reactions 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 .
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 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 of the PyroMark Q24 System”, page 24.

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 EGFR 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 PCR Reagents Supplied with the EGFR Pyro Kit

This protocol is for 4 separate PCR amplifications of regions containing codon 719 (exon 18), codons 768 and 790 (exon 20), codons 858–861 (exon 21), or deletions and complex mutations in exon 19 using the EGFR Pyro Primers.

Important points before starting

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

Things to do before starting

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

Procedure

- 1. Thaw all necessary 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 EGFR 719 or PCR Primer EGFR Ex 19 Del or PCR Primer EGFR 768 and 790 or PCR Primer EGFR 858–861	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 System.

Things to do before starting

- Allow all required reagents and solutions to reach room temperature (15–25°C) before starting.

Procedure

1. Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.
2. Prepare a master mix for DNA immobilization according to Table 5. Prepare a volume 10% greater than that required for the total number of reactions to be performed.

Table 5. Master mix for DNA immobilization

Component	Volume/sample
Streptavidin Sepharose High Performance	2 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. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.

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 5 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 (codon 719 [exon 18], codons 768 and 790 [exon 20], codons 858–861 [exon 21], or exon 19).

Things to do before starting

- Place the PyroMark Q24 Plate Holder on a heating block at 80°C for use in step 17.
- PyroMark Wash Buffer is supplied as a 10x concentrate. Before using for the first time, dilute to a 1x working solution by adding 225 ml high-purity water to 25 ml 10x PyroMark Wash Buffer (final volume of 250 ml).

Procedure

1. **Dilute a sufficient amount of each sequencing primer (Seq Primer EGFR 719, Seq Primer EGFR 768, Seq Primer EGFR 790, Seq Primer EGFR 858–861, and Seq Primer EGFR Exon 19 Del) 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 EGFR 719 or Seq Primer EGFR Ex 19 Del or Seq Primer EGFR 768 or Seq Primer EGFR 790 or Seq Primer EGFR 858–861	0.8 μ l	8 μ l
PyroMark Annealing Buffer	24.2 μ l	242 μ l
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).**

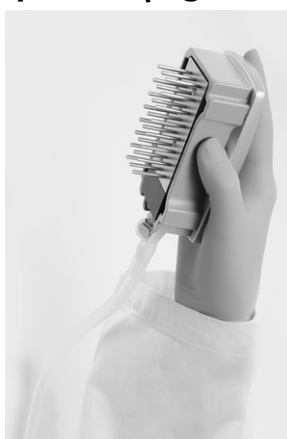


Figure 3. Illustration of the vacuum tool raised to beyond 90° vertical.

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 39.

- 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 of the PyroMark Q24 System”, page 24.**

Protocol 5: Running the PyroMark Q24 System

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

Important point before starting

- The Pre Run information report, found in the “Tools” menu at run setup (see “Protocol 1: Run Setup for the PyroMark Q24 System”, page 13), provides information about the volume of nucleotides, enzyme, and substrate buffer needed for a specific run.

Things to do before starting

- Switch on the PyroMark Q24. The power switch is located at the rear of the instrument.

Procedure

1. **Open the Buffers and Reagents box and remove the vials containing freeze-dried enzyme and substrate mixtures, and the vials containing nucleotides.**
2. **Dissolve the freeze-dried enzyme and substrate mixtures in 620 μ l each of water (supplied).**
3. **Mix by swirling the vial gently.**

Note: Do not vortex!

In order to ensure that the mixture is fully dissolved, leave it at room temperature (15–25°C) for 5–10 min. 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.

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

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

* 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.

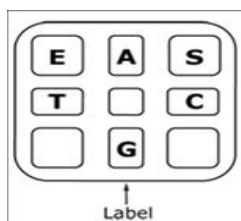


Figure 4. Illustration of the PyroMark Q24 Cartridge seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (**E**), substrate mixture (**S**), and nucleotides (**A**, **T**, **C**, **G**) according to the volume information given in the Pre Run information report, found in the “Tools” menu at run setup.

7. **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.**
8. **Ensure the line is visible in front of the cartridge and close the gate.**
9. **Open the plate-holding frame and place the plate on the heating block.**
10. **Close the plate-holding frame and the instrument lid.**
11. **Insert the USB stick (containing the run file) into the USB port at the front of the instrument.**
Note: Do not remove the USB port before the run is finished.
12. **Select “Run” in the main menu (using the ▲ and ▼ screen buttons) and press “OK”.**
13. **Select the run file using the ▲ and ▼ screen buttons.**
Note: To view the contents of a folder, select the folder and press “Select”. To go back to the previous view, press “Back”.
14. **When the run file is selected, press “Select” to start the run.**
15. **When the run is finished and the instrument confirms that the run file has been saved to the USB stick, press “Close”.**
16. **Remove the USB stick.**
17. **Open the instrument lid.**
18. **Open the cartridge gate and remove the reagent cartridge by lifting it up and pulling it out.**
19. **Close the gate.**
20. **Open the plate-holding frame and remove the plate from the heating block.**
21. **Close the plate-holding frame and the instrument lid.**
22. **Discard the plate and clean the cartridge, as per the instructions in the product sheet supplied with the cartridge.**
23. **Analyze the run according to “Protocol 6: Analysis of a PyroMark Q24 Run”, page 26.**

Protocol 6: Analysis of a PyroMark Q24 Run

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

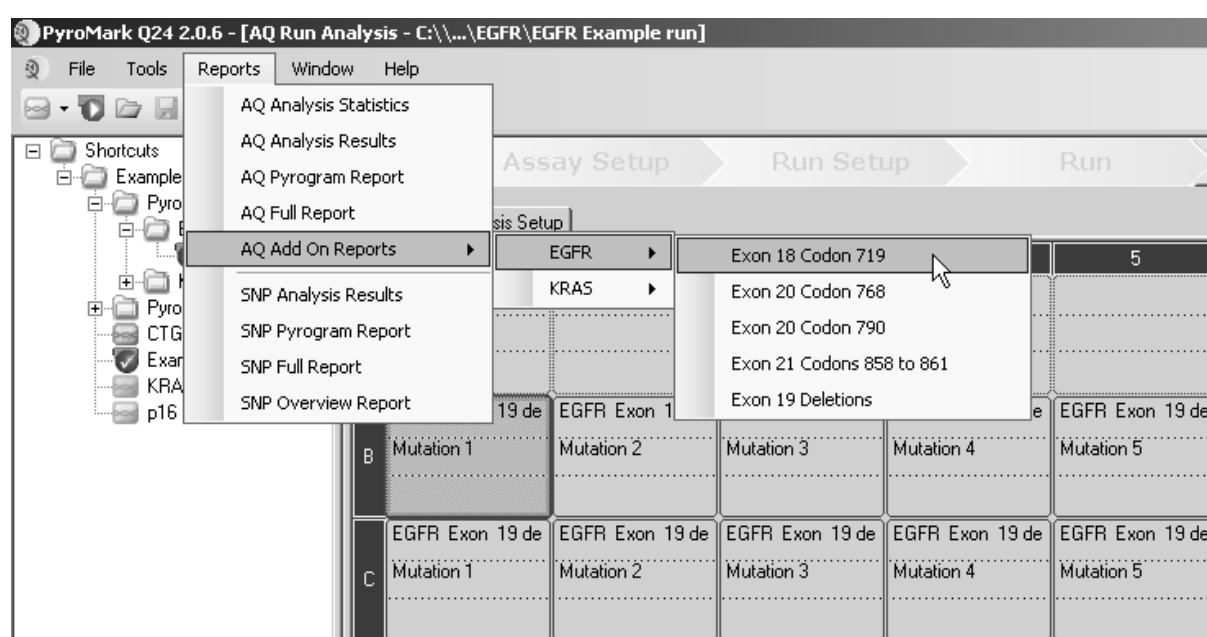
Procedure

1. Insert the USB stick containing the processed run file into the computer's USB port.
2. Move the run file from the USB stick to the desired location on the computer using Windows Explorer.
3. Open the run file in the AQ mode of PyroMark Q24 Software either by selecting "Open" in the "File" menu or by double-clicking the file (✓) in the shortcut browser.
4. There are 2 methods for analyzing the run. If using the EGFR Plug-in Report, go to step 5. If using the AQ analysis integral to the PyroMark Q24 System, go to step 6.

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

5. Using the EGFR Plug-in Report:

To generate a report, select "AQ Add On Reports/EGFR and "Exon 18 Codon 719" or "Exon 19 Deletions" or "Exon 20 Codon 768" or "Exon 20 Codon 790" or "Exon 21 Codons 858 to 961" from "Reports" in the menu.



The wells will automatically be analyzed for all mutations for which LOD is given in Table 7 (page 29). The results will be presented in an overview

table (see example below), followed by the detailed results containing, for example, Pyrograms and analysis quality.

Summary

Well	Sample ID	Result	Frequency	Amino Acid Substitution	Info
B1	Mutation 1	Mutation (2235del15)	5.5 %	del E746-A750	
B2	Mutation 2	Mutation (2236del15)	5.5 %	del E746-A750	
B3	Mutation 3	Mutation (2240del18)	5.3 %	del L747-P753insS	
B4	Mutation 4	Mutation (2240del15)	10.4 %	del L747-T751	
B5	Mutation 5	Mutation (2239_2248>C)	11.3 %	del L747-A750insP	
B6	Mutation 6	Mutation (2237_2255>T)	9.1 %	del E746-S752insV	
B8	WT	Wildtype			
C1	Mutation 1	Mutation (2235del15)	8.1 %	del E746-A750	
C2	Mutation 2	Mutation (2236del15)	9.2 %	del E746-A750	
C3	Mutation 3	Mutation (2240del18)	7.5 %	del L747-P753insS	
C4	Mutation 4	Mutation (2240del15)	16.6 %	del L747-T751	
C5	Mutation 5	Mutation (2239_2248>C)	15.6 %	del L747-A750insP	
C6	Mutation 6	Mutation (2237_2255>T)	13.6 %	del E746-S752insV	
C8	WT	Wildtype			

6. Using AQ analysis:

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



Analyze all wells.



Analyze the selected well.

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

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

The standard “Sequence to Analyze” as defined in the Analysis Setup addresses the most frequent mutations in codons 719, 768, 790, 858 and 861 and the most frequent deletion in exon 19 (see Appendix A, page 35). If a sample contains a less frequent mutation, the “Sequence to Analyze” can be changed to analyze also the mutation status at this position, as described in Appendix A.

Updated frequencies of mutations in the human EGFR gene are provided online by the Sanger Institute at www.sanger.ac.uk/genetics/CGP/cosmic/.

We recommend single peak heights above 20 RLU for the codon 768 assay and 30 RLU for the remaining 4 assays. Set 20 or 30 RLU, respectively, as the “required peak height for passed quality” in assay setup (see Appendix A and the *PyroMark Q24 User Manual*).

To allow proper quantification of codon 719 and codon 790, adjust the heights of the histogram bars in the assay setup (see Appendix A, page 35).

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 with standard "Sequence to analyze" or with "Check" or "Failed" quality assessment

We strongly recommend reanalyzing all samples with no mutation detected with the standard "Sequence to Analyze", as well as samples that received "Check" or "Failed" quality assessment. "Check" and "Failed" quality assessments may indicate a mutation in another position resulting in unexpected reference peaks. To reanalyze and target less frequent mutations go to "Analysis Setup" and change "Sequence to Analyze" to variants described in Appendix A. Click "Apply", and then click "To All" when the "Apply Analysis Setup" window appears.

Note: Ensure the threshold for single peak height and the height of histogram bars are adjusted accordingly (see Appendix A, page 35).

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 higher than the corresponding position in the normal sample should be examined in relation to the table showing the limit of detection (see Table 7, page 29). If using the EGFR Plug-in Report, this is performed automatically.

As a guide, samples that have a suspected mutation in the range from LOD (Table 7) to LOD + 3 % units should be reanalyzed in duplicate together with a normal sample in duplicate. If using the Plug-in Report (step 5), a warning will be issued if this occurs. If both duplicates give the same result as the original analysis and are visibly different from the normal control, then the sample can be considered to be positive for the mutation.

Table 7. LOD determined for specific mutations

Mutation	LOD (% units)	COSMIC ID* (V47)
Exon 19 deletions		
2233del15	1.6	26038
2235_2248>AATTC	1.6	13550
2235_2252>AAT	2.8	13551
2235del15	1.8	6223
2236del15	1.2	6225
2237_2252>T	2.4	12386
2237_2255>T	1.6	12384
2237del15	1.9	12678
2237del18	1.7	12367
2238_2248>GC	2.5	12422
2238_2252>GCA	0.6	12419
2238del18	1.1	6220
2239_2248>C	2.4	12382
2239_2251>C	1.7	12383
2239_2258>CA	3.9	12387
2239del18	1.5	6255
2239del9	3.7	6218
2240del12	1.5	6210
2240del15	1.9	12369
2240del18	1.9	12370

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

Table continued on next page.

Table 7. Continued

Mutation	LOD (% units)	COSMIC ID* (V47)
Exon 18 codon 719 (GGC)		
AGC	1.5	6252
TGC	1.6	6253
GCC	9.1	6239
Exon 20 codon 768 (AGC)		
ATC	5.0	6241
Exon 20 codon 790 (ACG)		
ATG	10.7	6240
Exon 21 codon 858 (CTG)		
CGG	2.6 (5.5) [†]	6224
Exon 21 codon 861 (CTG)		
CAG	4.3	6213
CGG	4.2	12374

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

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

Representative results

Representative Pyrogram results are shown in Figures 5–10.

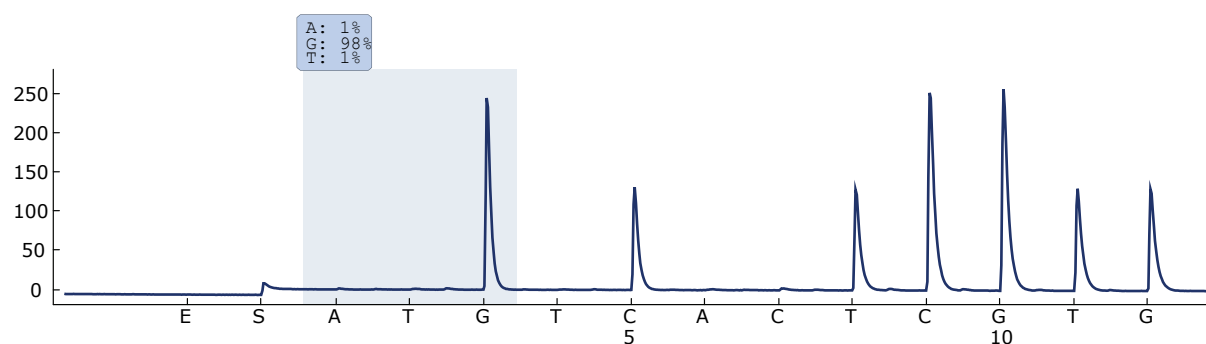


Figure 5. Pyrogram trace obtained after analysis of a sample with a normal genotype in codon 719 with the “Sequence to Analyze” *DGCTCCGGTGC*.

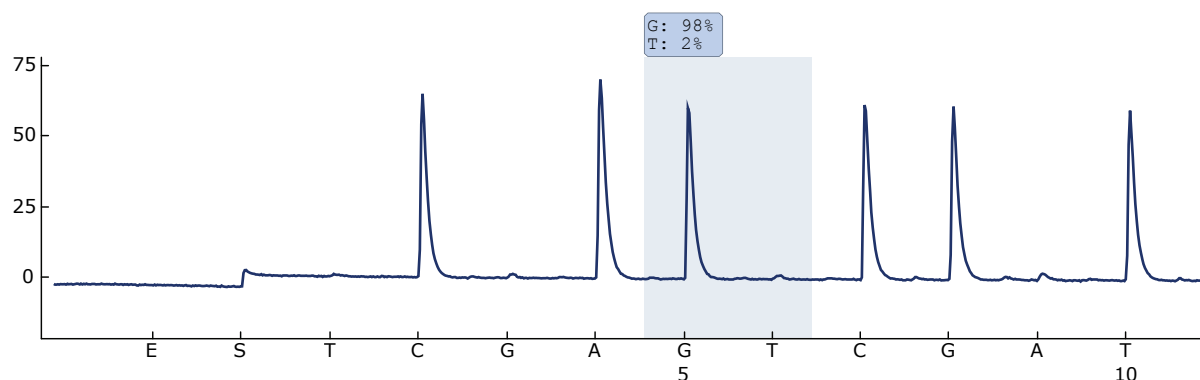


Figure 6. Pyrogram trace obtained after analysis of a sample with a normal genotype in codon 768 with the “Sequence to Analyze” *CAKCGTG*.

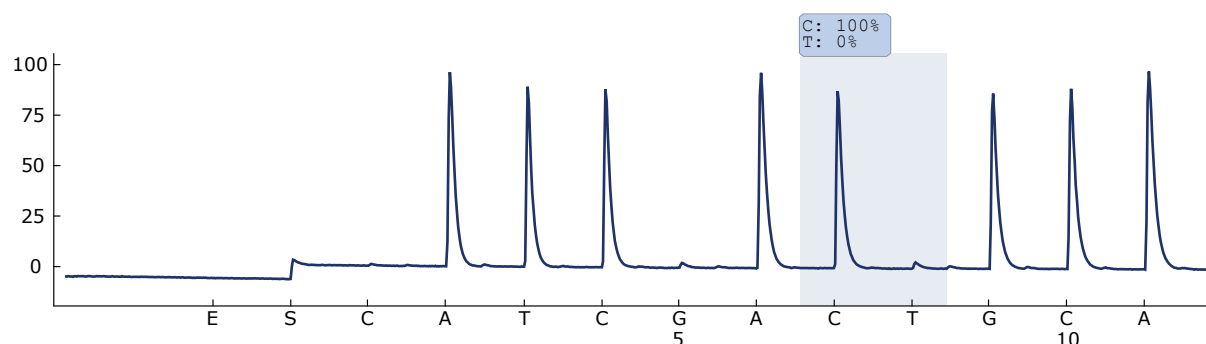


Figure 7. Pyrogram trace obtained after analysis of a sample with a normal genotype in codon 790 with the “Sequence to Analyze” *ATCAYGCAG*.

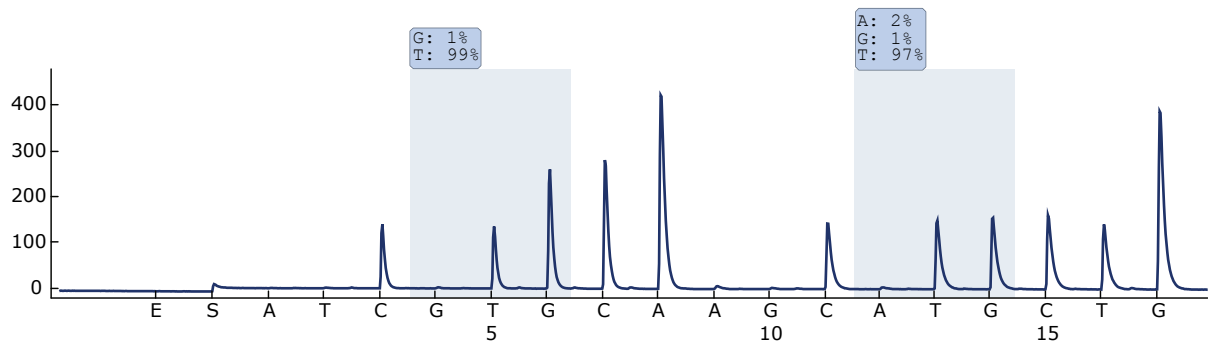


Figure 8. Pyrogram trace obtained after analysis of a sample with a normal genotype in codons 858–861 with the “Sequence to Analyze” CKGGCCAAACDGCTGGGT.

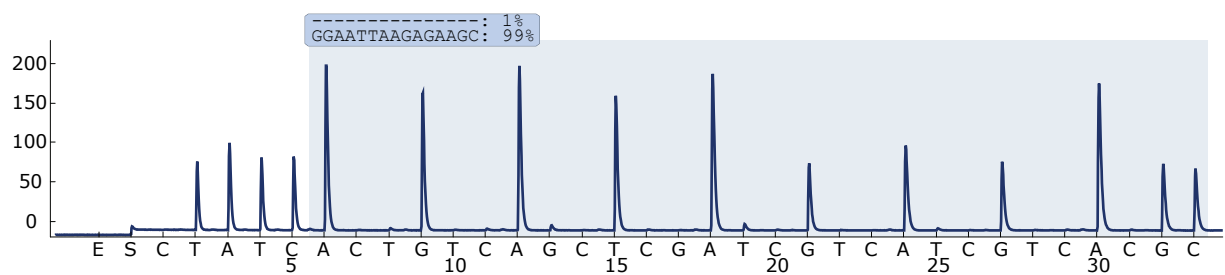


Figure 9. Pyrogram trace obtained after analysis of a sample with a normal genotype in exon 19.

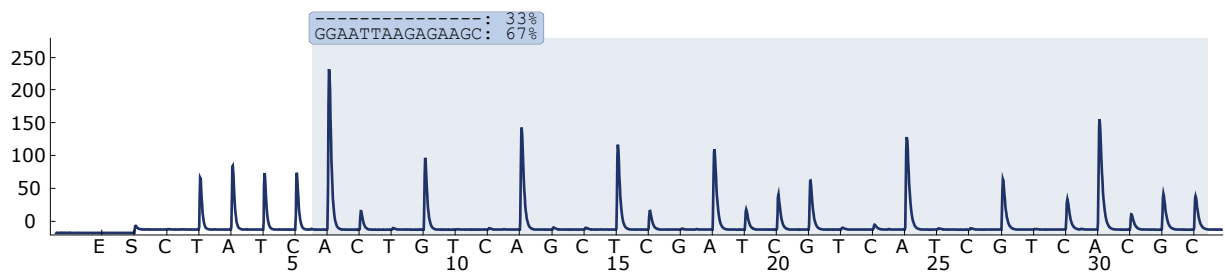


Figure 10. Pyrogram trace obtained after analysis of samples with a GGAATTAAGAGAAGC deletion in exon 19.

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, 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 35), 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. |
| c) Warning message
"Uncertain/Failed due to high peak height deviation at dispensation: 8" is appearing in the codon 790 assay | Background noise at dispensation T8 is below expected level. Adjust the height of the histogram bar to the default value (1.00) (only possible when using the analysis tool integral to the PyroMark Q24 System). |

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 EGFR Pyro Assays

If the EGFR Plug-in Report has been installed, use the assay setups supplied for codons 719, 768, 790, and 858–861 and exon 19 deletions. The following steps do not need to be performed. The EGFR Plug-in Report can be obtained from pyro.plugin@qiagen.com. We strongly recommend the use of the EGFR Plug-in Report over manual analysis. Complex mutations can not be added manually to a “Sequence to Analyze” and have to be analyzed using the plug-in. After installation of the plug-in or each time new software is installed or upgraded on the computer, the correct function of the plug-in should be verified as described in the EGFR Plug-In Quick Guide.

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

Procedure

Set up the assay for EGFR codon 719 by using the PyroMark Q24 Software.

EGFR codon 719

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

A2. Type the following sequence in “Sequence to Analyze”:
DGCTCCGGTGC

The most frequent mutations in codon 719 will be detected in nucleotide 2155 using this “Sequence to Analyze”.

The “Sequence to Analyze” can be changed after the run to analyze for mutations at nucleotide 2156.

To check if mutations are present at nucleotide 2156, change the “Sequence to Analyze” to the following sequence:

GSCTCCGGTGC

Note: Ensure the threshold for single peak height is set to 30 RLU. In addition, ensure that the heights of histogram bars are adjusted correctly (see instructions below).

A3. Manually enter the following “Dispensation Order”:
ATGTCAC TCGTG

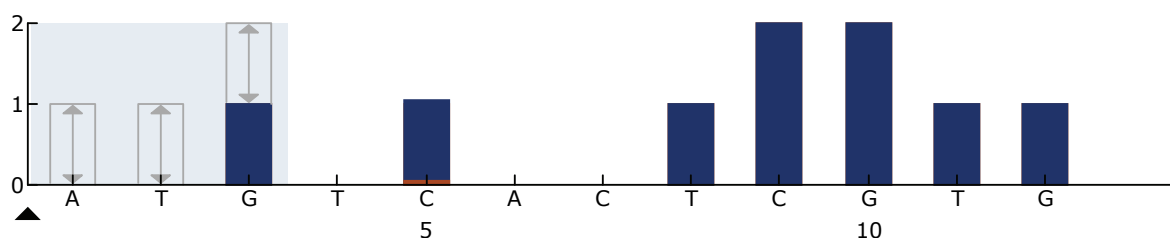


Figure 11. Histogram for codon 719 (nucleotide 2155) with the "Sequence to Analyze" *DGCTCCGGTGC*. The red rectangle at the bottom of the bar at dispensation C5 illustrates the adjustment of heights of histogram bar.

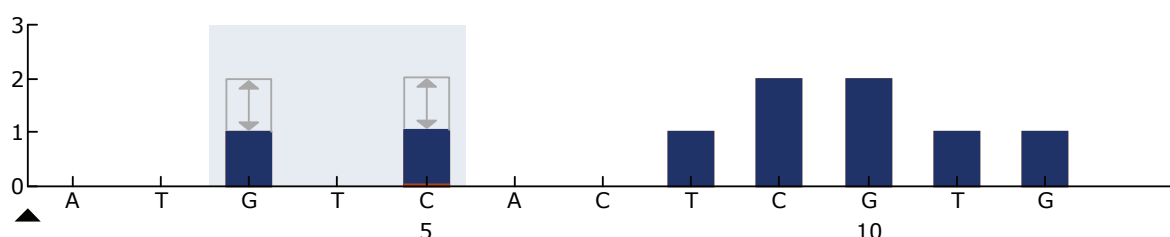


Figure 12. Histogram for codon 719 (nucleotide 2156) with the "Sequence to Analyze" *GSCTCCGGTGC*. The red rectangle at the bottom of the bar at dispensation C5 illustrates the adjustment of heights of histogram bar.

- A4. Click the "Analysis Parameters" tab, and increase "Peak Height Threshold - Required peak height for Passed quality:" to 30.**
- A5. In the histogram, move the mouse cursor at the upper end of the bar, at dispensation C5, and click while holding down the "Ctrl" button. A small window will appear showing the default height of the histogram bar (1.00). Increase the level to 1.04 for "Sequence to Analyze" *DGCTCCGGTGC* and to 2.04 for "Sequence to Analyze" *GSCTCCGGTGC*.**
- A6. Click  in the toolbar, and save the assay as "EGFR codon 719".**

EGFR codon 768

- A1. Click  in the toolbar and select "New AQ Assay".**
- A2. Type the following sequence in "Sequence to Analyze":**
CAKCGTG
- A3. Manually add the following "Dispensation Order":**
TCGAGTCGAT

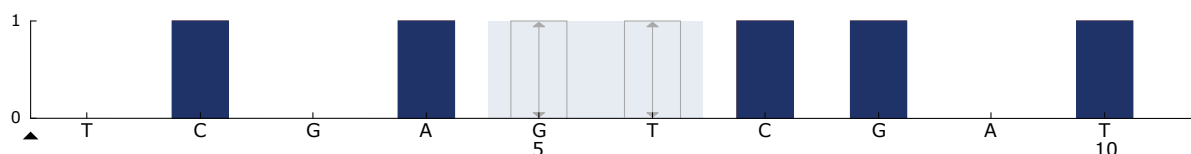


Figure 13. Histogram for codon 768 (nucleotide 2303) with the "Sequence to Analyze" CAKCGTG.

A4. Click  in the toolbar, and save the assay as "EGFR codon 768".

EGFR codon 790

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

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

ATCAYGCAG

A3. Manually add the following "Dispensation Order":

CATCGACTGCA

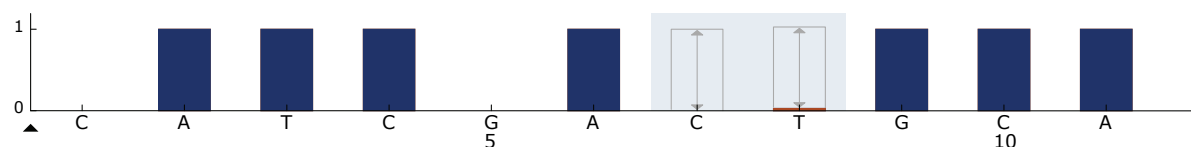


Figure 14. Histogram for codon 790 (nucleotide 2369) with the "Sequence to Analyze" ATCAYGCAG. The red rectangle at the bottom of the bar at dispensation T8 illustrates the adjustment of heights of histogram bar.

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

A5. In the histogram, move the mouse cursor at the upper end of the bar at dispensation T8, and click twice while holding down the "Ctrl" button. A small window will appear showing the default height of the histogram bar (1.00). Increase the level to 1.03.

A6. Click  in the toolbar, and save the assay as "EGFR codon 790".

EGFR codons 858–861

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

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

CKGGCCAAACDGCTGGGT

A3. Manually add the following "Dispensation Order":

ATCGTGCAAGCATGCTG

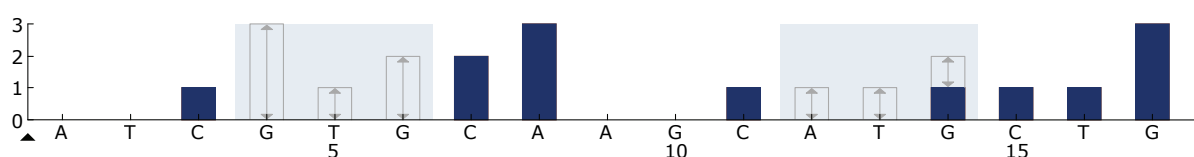


Figure 15. Histogram for codons 858–861 with the “Sequence to Analyze” CKGGCCAAACDGCTGGGT.

- 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 “EGFR codons 858–861”.**

EGFR Exon 19 del

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

A2. Type the following sequence in “Sequence to Analyze”:

TATCAA[GGAATTAAGAGAAGC]AACATCTCCGAAAGCCAACAAGGA

The most frequent deletion in exon 19 is 2235del15. To analyze for other deletions, the “Sequence to Analyze” has to be changed according to each defined deletion.

Use the wt sequence

**TATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAA
ATCCTCGAT** and add squared brackets where the deletion starts and ends.

For the second most frequent deletion in exon 19 (2236del15), change “Sequence to Analyze” to the following:

TATCAAG[GAATTAAGAGAAGCA]ACATCTCCGAAAGCCAACAAGGA

A3. Manually add the following “Dispensation Order”:

CTATCACTGTCAGCTCGATCGTCATCGTCACGC

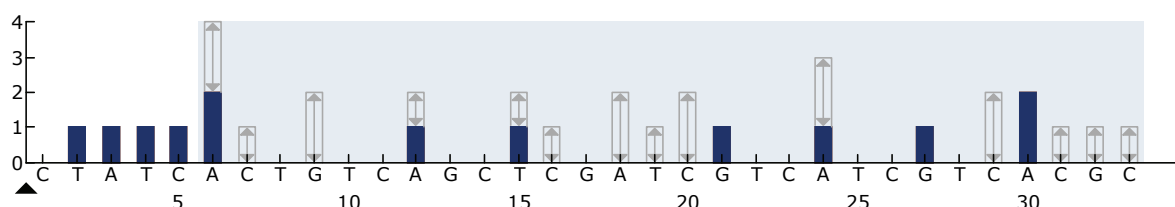


Figure 16. Histogram for exon 19 del.

- 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 “EGFR Exon 19 del”.**

Appendix B: Emptying the Waste Container and Troughs

WARNING 	Hazardous chemicals The Denaturation Solution used with the vacuum workstation contains sodium hydroxide, which is irritating to eyes and skin. Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Material Safety Data Sheets (MSDSs) or OSHA,* ACGIH,[†] or COSHH[‡] documents. Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.
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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 (Milli-Q 18.2 MΩ x cm, www.millipore.com, or equivalent).

Procedure

- B1. Ensure that no vacuum is applied to the vacuum tool. Make sure that the vacuum is closed (Off) and the vacuum pump is switched off.**
- B2. Discard any solutions left in the troughs.**
- B3. Rinse the troughs with high-purity water, or replace them if necessary.**
- B4. Empty the waste container.**
The cap can be removed without disconnecting the tubing.
- B5. If the vacuum workstation must be cleaned (for example, due to dust or spillage), follow the instructions in the *PyroMark Q24 User Manual*.**

References

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

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

Ordering Information

Product	Contents	Cat. no.
EGFR 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	970480
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 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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Brazil ■ Orders 0800-557779 ■ Fax 55-11-5079-4001 ■ Technical 0800-557779

Canada ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

China ■ Orders 86-21-3865-3865 ■ Fax 86-21-3865-3965 ■ Technical 800-988-0325

Denmark ■ Orders 80-885945 ■ Fax 80-885944 ■ Technical 80-885942

Finland ■ Orders 0800-914416 ■ Fax 0800-914415 ■ Technical 0800-914413

France ■ Orders 01-60-920-926 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930 ■ Offers 01-60-920-928

Germany ■ Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

Hong Kong ■ Orders 800 933 965 ■ Fax 800 930 439 ■ Technical 800 930 425

Ireland ■ Orders 1800 555 049 ■ Fax 1800 555 048 ■ Technical 1800 555 061

Italy ■ Orders 800-789-544 ■ Fax 02-334304-826 ■ Technical 800-787980

Japan ■ Telephone 03-6890-7300 ■ Fax 03-5547-0818 ■ Technical 03-6890-7300

Korea (South) ■ Orders 080-000-7146 ■ Fax 02-2626-5703 ■ Technical 080-000-7145

Luxembourg ■ Orders 8002-2076 ■ Fax 8002-2073 ■ Technical 8002-2067

Mexico ■ Orders 01-800-7742-639 ■ Fax 01-800-1122-330 ■ Technical 01-800-7742-436

The Netherlands ■ Orders 0800-0229592 ■ Fax 0800-0229593 ■ Technical 0800-0229602

Norway ■ Orders 800-18859 ■ Fax 800-18817 ■ Technical 800-18712

Singapore ■ Orders 1800-742-4362 ■ Fax 65-6854-8184 ■ Technical 1800-742-4368

Spain ■ Orders 91-630-7050 ■ Fax 91-630-5145 ■ Technical 91-630-7050

Sweden ■ Orders 020-790282 ■ Fax 020-790582 ■ Technical 020-798328

Switzerland ■ Orders 055-254-22-11 ■ Fax 055-254-22-13 ■ Technical 055-254-22-12

UK ■ Orders 01293-422-911 ■ Fax 01293-422-922 ■ Technical 01293-422-999

USA ■ Orders 800-426-8157 ■ Fax 800-718-2056 ■ Technical 800-DNA-PREP (800-362-7737)

