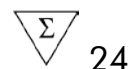


***therascreen*[®] EGFR Pyro[®] Kit Handbook**



Version 1



For in vitro diagnostic use



971480



1061827EN



QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, GERMANY

R3

MAT

1061827EN



QIAGEN Sample and Assay Technologies

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

QIAGEN sets standards in:

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

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

Contents

Intended Use	5
Summary and Explanation	5
Principle of the Procedure	6
Materials Provided	8
Kit contents	8
Materials Required but Not Provided	10
Warnings and Precautions	11
Safety information	11
General precautions	12
Reagent Storage and Handling	13
Specimen Handling and Storage	13
Procedure	14
DNA isolation	14
Protocols	
■ 1: Run setup for the PyroMark Q24 system	15
■ 2: PCR using the PCR reagents supplied with the <i>therascreen</i> EGFR Pyro Kit	17
■ 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads	20
■ 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24	22
■ 5: Running the PyroMark Q24	26
■ 6: Analysis of a PyroMark Q24 run	28
Interpretation of Results	31
Interpretation of analysis results and detection of low-level mutations	31
Troubleshooting guide	36
Quality Control	39
Limitations	39
Performance Characteristics	40
Limit of blank and limit of detection	40
Linearity	43
Precision	43

Diagnostic evaluation	44
References	49
Symbols	50
Contact Information	50
Appendix A: Setting Up <i>therascreen</i> EGFR Pyro Assays	51
Appendix B: Emptying the Waste Container and Troughs	55
Ordering Information	56

Intended Use

The *therascreen* EGFR Pyro Kit is an *in vitro* nucleic acid sequence-based detection test based on Pyrosequencing® for the quantitative detection of mutations in exons 18, 19, 20, and 21 of the human EGFR gene in genomic DNA derived from human tissue samples.

The *therascreen* EGFR Pyro Kit is intended to provide clinicians with information to aid the selection of cancer patients more likely to benefit from treatment with anti-EGFR therapies. For *in vitro* diagnostic use.

For use only on the PyroMark® Q24 system. PyroMark Q24 systems include the following:

- The PyroMark Q24 instrument and the PyroMark Q24 MDx instrument.
- The PyroMark Q24 Vacuum Workstation and the PyroMark Q24 MDx Vacuum Workstation.
- PyroMark Q24 Software (version 2.0) and PyroMark Q24 MDx Software (version 2.0).

The product is intended to be used by professional users, such as technicians and physicians who are trained in *in vitro* diagnostics procedures, molecular biological techniques, and the PyroMark Q24 system.

Summary and Explanation

The *therascreen* EGFR Pyro Kit enables quantitative measurement of mutations in codons 719, 768, 790, and 858–861, as well as deletions and complex mutations in exon 19 of the human EGFR gene.

The kit consists of four PCR assays (Figure 1) for the detection of:

- Mutations in codon 719 (exon 18)
- Mutations in codons 768 and 790 (exon 20)
- Mutations in codons 858 to 861 (exon 21)
- Deletions and complex mutations in exon 19

The four regions are amplified separately by PCR and sequenced through the defined region. The amplicon covering codons 768 and 790 is divided into two sequencing reactions. Sequences surrounding the defined positions serve as normalization and reference peaks for quantification and quality assessment of the analysis.

All assays are sequenced in the forward direction.

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

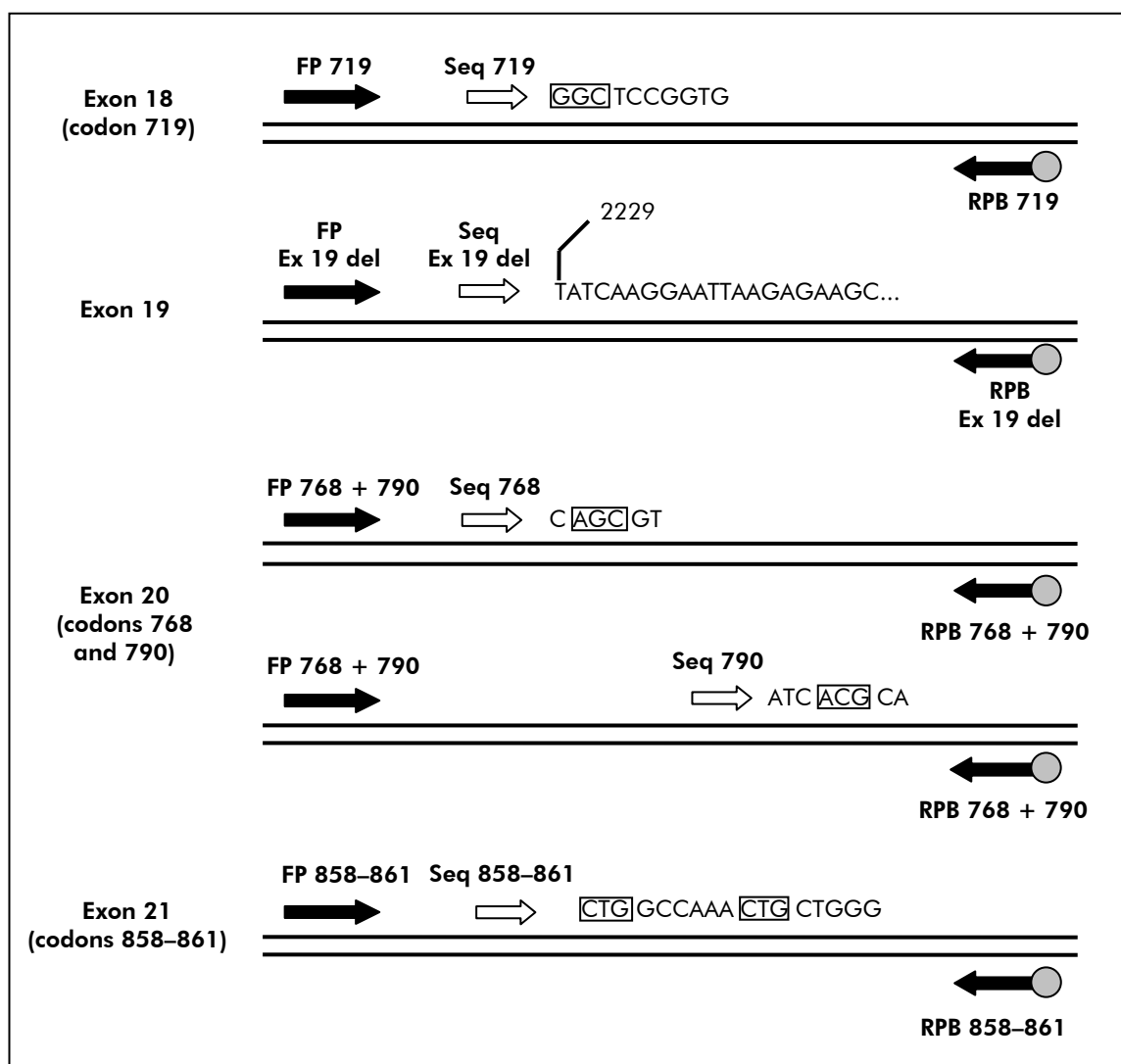


Figure 1. Illustration of the EGFR assay. The sequence indicated is the analyzed sequence for a wild-type sample. **FP:** Forward PCR primers; **RPB:** Reverse PCR primers (B indicates biotinylation); **Seq:** Sequencing primers.

Principle of the 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.

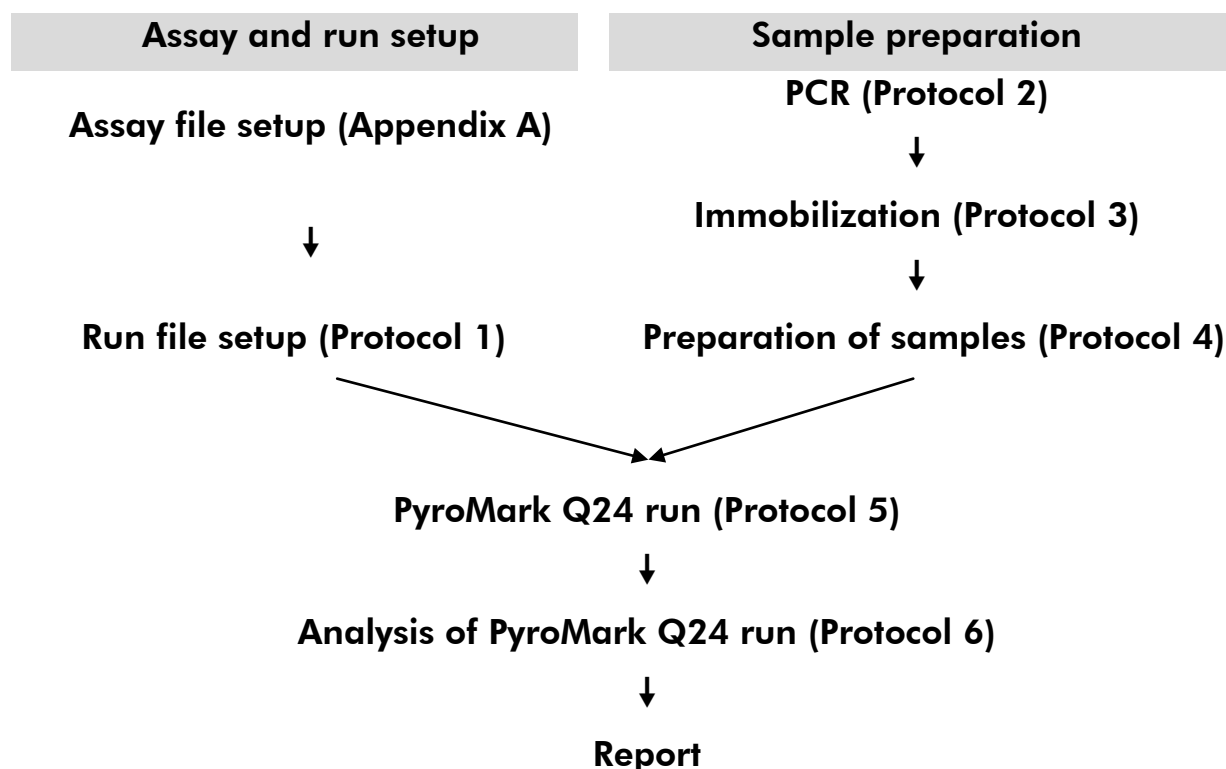
It is recommended to use the EGFR Plug-in Report to analyze the run. The EGFR Plug-in Report can be obtained by e-mail from pyro.plugin@qiagen.com.

However, the run can also be analyzed using the analysis tool integral to the PyroMark Q24 system. The "Sequence to Analyze" can then be 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 28).

Note: The workflow has been slightly modified compared to revision R1 of the *therascreen EGFR Pyro Kit Handbook* (see “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 22).

Workflow of *therascreen EGFR 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 “Interpretation of Results”, page 31). 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

therascreen EGFR Pyro Kit (box 1/2)

<i>therascreen</i> EGFR Pyro Kit	(24)
Catalog no.	971480
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

therascreen buffers and reagents (box 2/2)

<i>therascreen</i> 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.

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 14)
- 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. 9001513 or 9001514)*†
- PyroMark Q24 Software (cat. no. 9019063 or 9019062)†
- PyroMark Q24 Plate (cat. no. 979301)†
- PyroMark Q24 Cartridge (cat. no. 979302)†
- PyroMark Q24 Vacuum Workstation (cat. no. 9001515 or 9001517)*†
- 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, 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%)‡

* Ensure that instruments have been checked and calibrated according to the manufacturer's recommendations.

† CE-IVD-marked in accordance with EU Directive 98/79/EC. All other products listed are not CE-IVD-marked based on EU Directive 98/79/EC.

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

Recommended plate mixers

The plate mixers shown in Table 1 are recommended for use with the *therascreen* EGFR Pyro Kit.

Table 1. Plate mixers recommended for use with the *therascreen* EGFR 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 0.2ml 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)

Warnings and Precautions

For In Vitro Diagnostic Use

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.

The following hazard and precautionary statements apply to components of the *therascreen EGFR Pyro Kit*.

PyroMark Denaturation Solution



Warning! Causes skin irritation. Causes serious eye irritation. May be corrosive to metals. Absorb spillage to prevent material damage. Keep only in original container. Wear protective gloves/protective clothing/eye protection/face protection.

PyroMark Enzyme Mixture



Contains: (R*,R*)-1,4-Dimercaptobutane-2,3-diol; acetic acid. Danger! Causes skin irritation. Causes serious eye damage. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. IF exposed or concerned: Call a POISON CENTER or doctor/physician. Take off contaminated clothing and wash it before reuse. Wear protective gloves/protective clothing/eye protection/face protection.

PyroMark Substrate Mixture



Contains: acetic acid. Warning! Causes skin irritation. Causes serious eye irritation. If eye irritation persists: Get medical advice/attention. Take off contaminated clothing and wash it before reuse. Wear protective gloves/protective clothing/eye protection/face protection.

General precautions

Note: 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.
- The workflow has been slightly modified (see “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 22) compared to revision R1 of the *therascreen EGFR Pyro Kit Handbook*.
- 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 *therascreen* EGFR Pyro Kit is shipped in two boxes. The *therascreen* 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 –30°C to –15°C upon arrival.

The *therascreen* buffers and reagents (box 2/2) containing buffers, enzyme mixture, substrate mixture, dATP α S, dCTP, dGTP, and dTTP (the reagents for Pyrosequencing[®] analysis) are 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 –30°C to –15°C. Frozen reagents should not be subjected to more than 3 freeze–thaw cycles.

Note: Nucleotides should not be frozen.

The *therascreen* EGFR 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 system performance has been established using the EZ1[®] DNA Tissue Kit and the QIAamp[®] DNA FFPE Tissue Kit for extraction of human DNA from formalin-fixed paraffin-embedded tumor samples. For the QIAamp DSP DNA Blood Mini Kit system, performance has been established using healthy donor blood samples partially spiked with tumor cells.

The QIAGEN[®] kits shown in Table 2 are recommended for DNA purification from the indicated human sample types for use with the *therascreen* EGFR Pyro Kit. Carry out the DNA purification according to the instructions in the kit handbooks.

Table 2. DNA purification kits recommended for use with the *therascreen* 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
Blood	QIAamp DSP DNA Blood Mini Kit [†]	61104

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

[†] CE-IVD marked in accordance with EU Directive 98/79/EC.

Protocol 1: Run setup for the PyroMark Q24 system

Important point before starting

- If required, the LOB can be confirmed by using a wild-type sample to generate a full plate of results. For details, consult CLSI Guideline EP17-A "Protocol for determination of limits of detection and limits of quantitation; approved guideline".

Things to do before starting

- If the EGFR Plug-in Report has not been installed, create an Assay Setup (see Appendix A, page 51). This must only be done once before running the *therascreen* EGFR Pyro assays for the first time. In case the EGFR Plug-in Report has been installed, predefined Assay Setups are available in the shortcut browser of the PyroMark Q24 software under the path "Example Files/PyroMark Setups/EGFR". The EGFR Plug-in Report can be obtained by e-mail from pyro.plugin@qiagen.com.

Procedure

1. Click in the toolbar.

A new run file is created.

2. Enter the run parameters (see "Run parameters", page 16).

3. Set up the plate by adding assays for the 5 different sequencing reactions 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 (see "Controls", page 11).

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.

Note: 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 20).

To run the plate on PyroMark Q24, see "Protocol 5: Running the PyroMark Q24", page 26.

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 <i>therascreen</i> EGFR Pyro Kit to be used. The lot number can be found on the product label. Note: 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 *therascreen* 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 *therascreen* EGFR Pyro primers.

Important points before starting

- The HotStarTaq[®] DNA polymerase in the PyroMark 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 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 to 0.4–2 ng/ μ l, if necessary.

Procedure

- 1. Thaw all necessary components (see Table 3).**
Mix well before use.
- 2. Prepare a reaction mix for each PCR primer set according to Table 3.**
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 3. 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 EGFR 719 or PCR Primer EGFR Ex19 Del or PCR Primer EGFR 768 and 790 or PCR Primer EGFR 858–861	1.0
Water (H ₂ O, supplied)	4.0
Total volume	20.0

3. Mix the reaction mix thoroughly and dispense 20 μl into each PCR tube.

It is not necessary to keep PCR tubes on ice since HotStarTaq DNA Polymerase is inactive at room temperature.

4. Add 5 μl template DNA (2–10 ng of genomic DNA) to the individual PCR tubes (see Table 4) and mix thoroughly.

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

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

Table 4. 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 5.

Table 5. 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 20.

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

Table 6. Master mix for DNA immobilization

Component	Volume/sample (μl)
Streptavidin Sepharose High Performance	2
PyroMark Binding Buffer	40
Water (H ₂ O, supplied)	28
Total volume	70

3. Add 70 μl of the master mix to wells of a 24-well PCR plate or strips as predefined in the run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 15).
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 1: Run setup for the PyroMark Q24 system”, page 15).
Note: The total volume per well should be 80 μl after addition of the master mix and PCR product.
5. Seal the PCR plate (or strips) using strip caps.
Note: 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.**

Note: 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 22.**

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

If more than 1 minute has elapsed since the plate (or strips) was agitated, agitate again for 1 minute 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 15), depending on the region of analysis (codon 719 [exon 18], codons 768 and 790 [exon 20], codons 858–861 [exon 21], or exon 19).
- The workflow has been slightly modified compared to revision R1 of the theascreen *EGFR Pyro Kit Handbook* (step 18). Do not shorten the time for cooling down the samples after heating to 80°C.
- 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.

Things to do before starting

- Place one PyroMark Q24 Plate Holder on a preheated heating block at 80°C for use in step 17. Leave a second PyroMark Q24 Plate Holder at room temperature (15–25°C) for use in step 18.
- 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.

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 7.**

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 7. Example dilution of the sequencing primers

Component	Volume/sample (μl)	Volume for 9 + 1 reactions (μl)
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	8
PyroMark Annealing Buffer	24.2	242
Total volume	25	250

- 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 15).**

Note: 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.

- Place the PCR plate (or strips) from Protocol 3 and the PyroMark Q24 Plate on the worktable (Figure 2).**

Note: 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 seconds. Take care when picking up the vacuum tool.

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

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

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

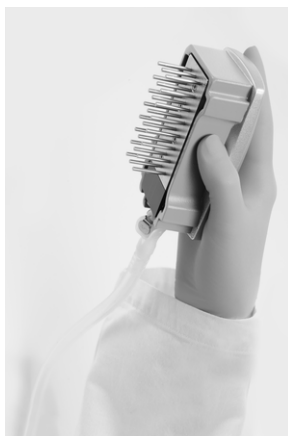


Figure 3. Illustration of the vacuum tool raised to beyond 90° vertical.

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 PyroMark Q24 Plate by lowering the filter probes into the diluted sequencing primer and moving the tool gently from side to side.
Note: Take care not to damage the surface of the PyroMark Q24 Plate by scratching it with the filter probes.
12. Transfer the vacuum tool to the trough containing high-purity water (Figure 2) and agitate it for 10 seconds.
13. Wash the filter probes by lowering the probes into high-purity water (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 seconds 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.**

Note: 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 55).
- 17. Heat the PyroMark Q24 Plate with the samples at 80°C for 2 minutes using the prewarmed PyroMark Q24 Plate Holder.**
- 18. 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.**
- 19. Proceed with “Protocol 5: Running the PyroMark Q24”, page 26.**

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

Note: Do not vortex!

Note: In order 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 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 safety data sheets (SDSs), available from the product supplier.

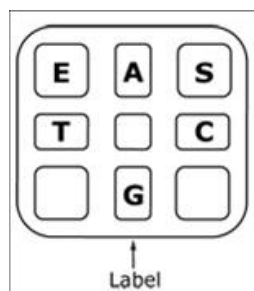


Figure 4. Illustration of the PyroMark Q24 Cartridge as seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (**E**), substrate mixture (**S**), and nucleotides (**A**, **T**, **C**, **G**) according to the volume information given in the Pre Run information 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.**
Note: 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.**
Note: 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 28.**

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, go to step 6.

Note: We strongly recommend using the EGFR Plug-in Report for result interpretation. The EGFR Plug-in Report can be obtained by e-mail from pyro.plugin@qiagen.com. This report ensures that the respective LOD values and different "Sequences to Analyze" are used to automatically detect all mutations and deletions, including the identification of twenty different deletions and complex mutations in Exon 19.

5. Using the EGFR Plug-in 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 (Figure 5).

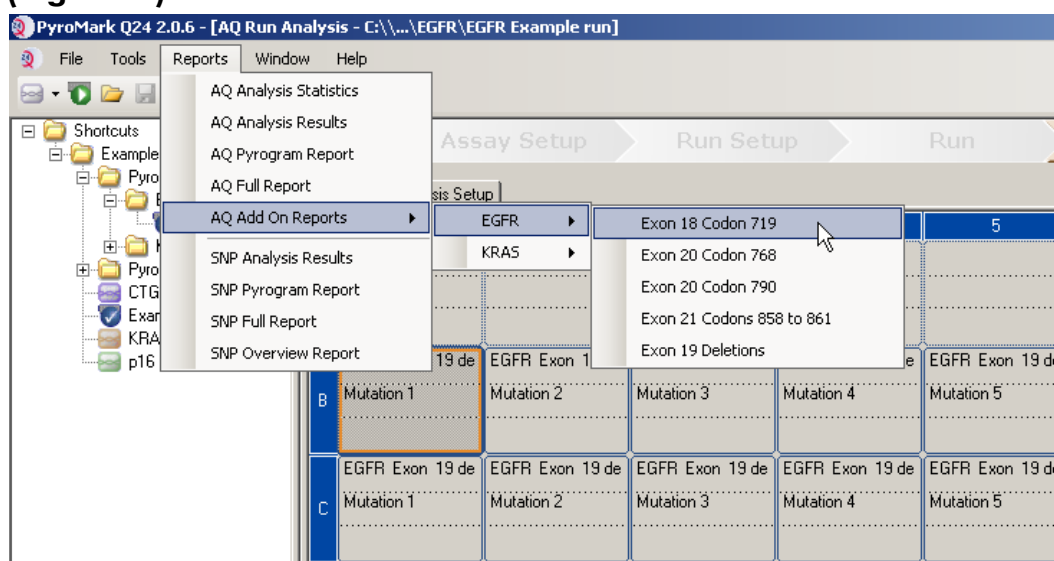


Figure 5. The AQ Run Analysis screen.

The wells will automatically be analyzed for all mutations for which the LOD is given in Table 8. The results will be presented in an overview table (Figure 6), followed by the detailed results which include Pyrograms and analysis quality.

Summary

Well	Sample ID	Result	Frequency [% units]	Amino Acid Substitution	Info
A1	B104683	Mutation	34.0	del E746-A750	
A2	B105072	Wildtype			
A3	B116390	Mutation	26.6	delL747-P753insS	
A4	B116389	Wildtype			
A5	B116301	Potential low level mutation	3.2	delK745-E749	!
A6	B116392	Mutation	15.4	del E746-A750	
A7	WT control	Wildtype			
A8	NTC	Failed Analysis			!

! See detailed results for further explanation.

NOTE: For further information about data evaluation please refer to the handbook.

Figure 6. The results summary table.

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*.

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

Note: 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 51). 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/.

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

Note: To allow proper quantification for codon 719 and codon 790, adjust the heights of the histogram bars in the assay setup (see Appendix A, page 51).

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 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 or variants for other rare or unexpected mutations. Click “Apply”, and then click “To All” when the “Apply Analysis Setup” window appears.

Note: After changing “Sequence to Analyze”, ensure the threshold for single peak height and the height of histogram bars are adjusted accordingly (see Appendix A, page 51).

Note: If the measured peaks do not match the height of the histogram bars and cannot be explained by rare or unexpected mutations, the result is not a basis for judgment of mutational status. It is recommended to rerun the sample.

Interpretation of Results

Interpretation of analysis results and detection of low-level mutations

It is strongly recommended that unmethylated control DNA is included in every run for comparison and as a control for background levels. The measured frequency of the control sample should be smaller or equal to the limit of blank (LOB).

All samples should be examined in relation to the limit of detection (LOD, see Table 8) and interpreted as follows.

- Mutation frequency $< \text{LOD}$: Wild type
- Mutation frequency $\geq \text{LOD}$ and $\leq \text{LOD} + 3 \%$ units: Potential low-level mutation

Note: If using the Plug-in Report (see step 5 of “Protocol 6: Analysis of a PyroMark Q24 run”, page 28) and this occurs, a warning will be issued.

Samples with a reported potential low-level mutation should only be considered positive for the mutation if confirmed by rerunning in duplicate together with a sample with unmethylated control DNA. The result of both duplicates should be $\geq \text{LOD}$ and different from the control sample.

Otherwise the sample should be judged as wild type.

- Mutation frequency $> \text{LOD} + 3 \%$ units: Mutation

If using the EGFR Plug-in Report, this is performed automatically.

Note: It is recommended to use the EGFR Plug-in Report for result interpretation. For closer examination of samples with a reported potential low-level mutation, we recommend to additionally analyze the sample manually in the application software (e.g., for comparison to the mutational frequency of the control sample).

Note: A measured frequency above LOB in the control sample indicates a higher than usual level of background in the respective run, which might impact allele quantification, especially for low mutational levels. In this case, measured frequencies in the range from LOD (Table 8) to $\text{LOD} + 3 \%$ units are not a basis for judgment of mutational status. It is recommended to rerun samples with a potential low-level mutation.

Note: A treatment decision for cancer patients must not be based solely on EGFR mutation status.

Table 8. LOB and LOD determined for specific mutations

Mutation	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V47)
Exon 19 deletions				
2233del15	K745_E749del	0.6	1.6	26038
2235_2248>AATTC	E746_A750>IP	0.8	1.6	13550
2235_2252>AAT	E746_T751>I	1.1	2.8	13551
2235del15 [†]	E746_A750del	0.9	1.8	6223
2236del15 [†]	E746_A750del	0.2	1.2	6225
2237_2252>T	E746_T751>V	0.8	2.4	12386
2237_2255>T [†]	E746_S752>V	0.6	1.6	12384
2237del15 [†]	E746_T751>A	0.9	1.9	12678
2237del18	E746_S752>A	0.5	1.7	12367
2238_2248>GC	L747_A750>P	0.8	2.5	12422
2238_2252>GCA	L747_T751>Q	0.2	0.6	12419
2238del18	E746_S752>D	0.3	1.1	6220
2239_2248>C [†]	L747_A750>P	1.8	2.5	12382
2239_2251>C	L747_T751>P	0.6	1.7	12383
2239_2258>CA	L747_P753>Q	1.3	3.9	12387
2239del18 [†]	L747_S752del	0.6	1.5	6255
2239del9	L747_E749del	2.0	3.7	6218
2240del12	L747_T751>S	0.4	1.5	6210
2240del15 [†]	L747_T751del	0.9	1.9	12369
2240del18 [†]	L747_P753>S	0.9	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.

[†] The LOD for these deletions in Exon 19 were determined by adding six standard deviations of blank measurements to the LOB value.

Table continued on next page

Table 8. Continued

Mutation	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V47)
Exon 18 codon 719 (GGC)				
AGC	G719S	0.9	1.5	6252
TGC	G719C	1.0	1.6	6253
GCC	G719A	4.7	9.1	6239
Exon 20 codon 768 (AGC)				
ATC	S768I	2.6	5.0	6241
Exon 20 codon 790 (ACG)				
ATG	T790M	7.0	10.7	6240
Exon 21 codon 858 (CTG)				
CGG	L858R	0.6	2.6 (5.5) [‡]	6224
Exon 21 codon 861 (CTG)				
CAG	L861Q	3.2	4.3	6213
CGG	L861R	1.9	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 7–14.

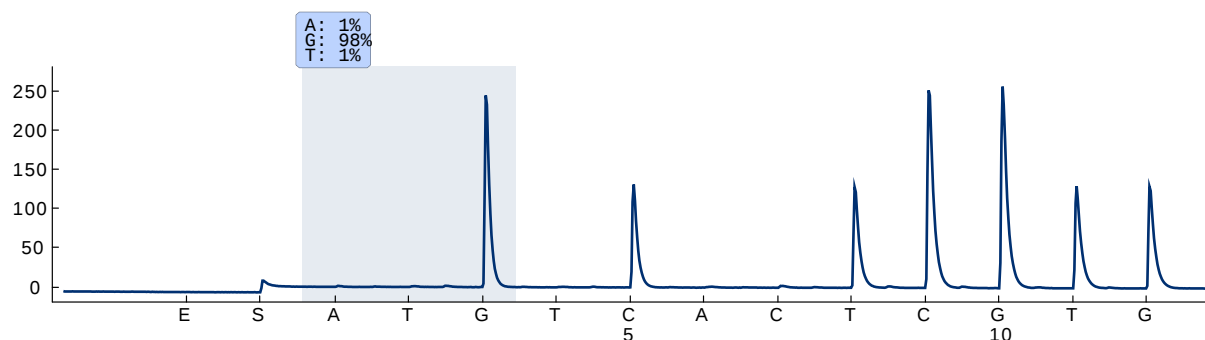


Figure 7. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codon 719 with the “Sequence to Analyze” DGCTCCGGTGC.

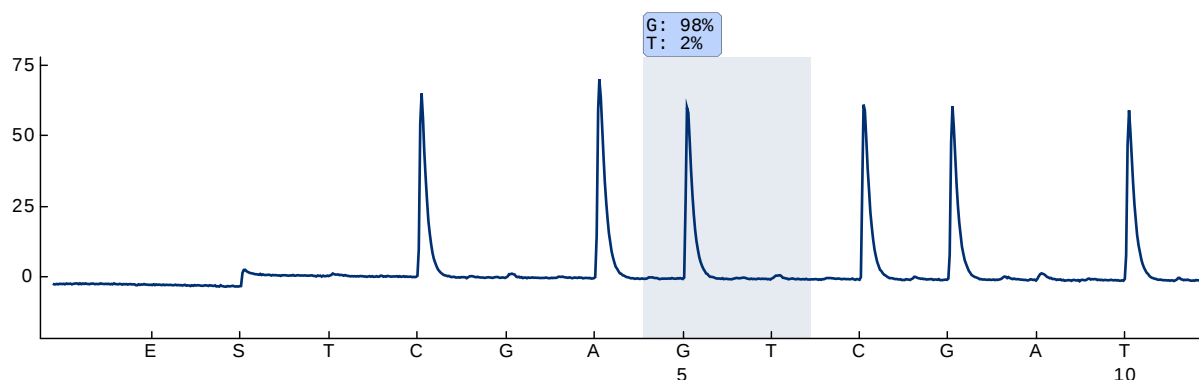


Figure 8. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codon 768 with the “Sequence to Analyze” CAKCGTG.

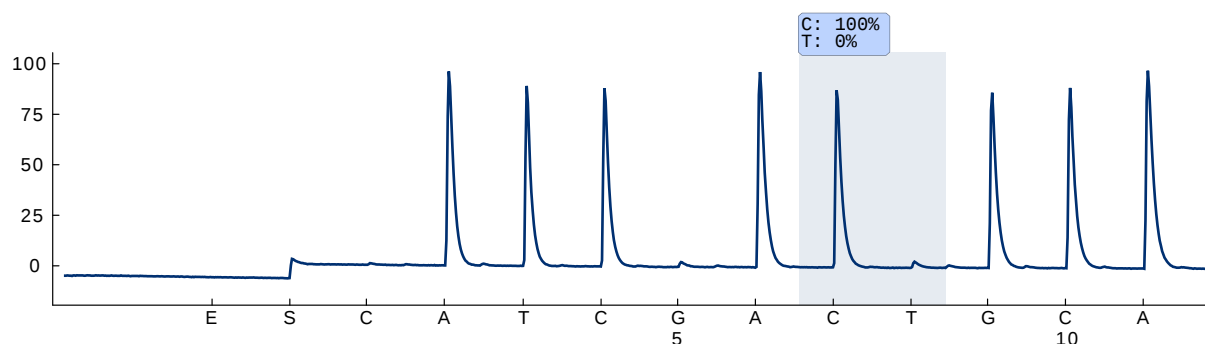


Figure 9. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codon 790 with the “Sequence to Analyze” ATCAYGCAG.

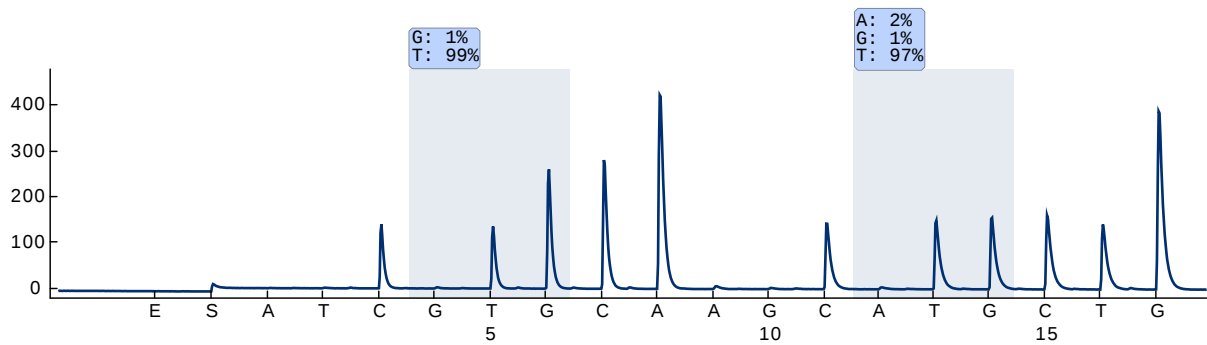


Figure 10. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codons 858–861 with the “Sequence to Analyze” CKGGCCAAACDGCTGGGT.

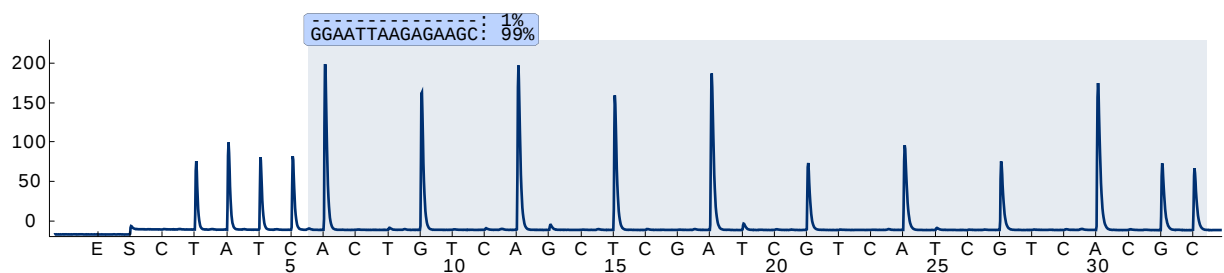


Figure 11. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in exon 19.

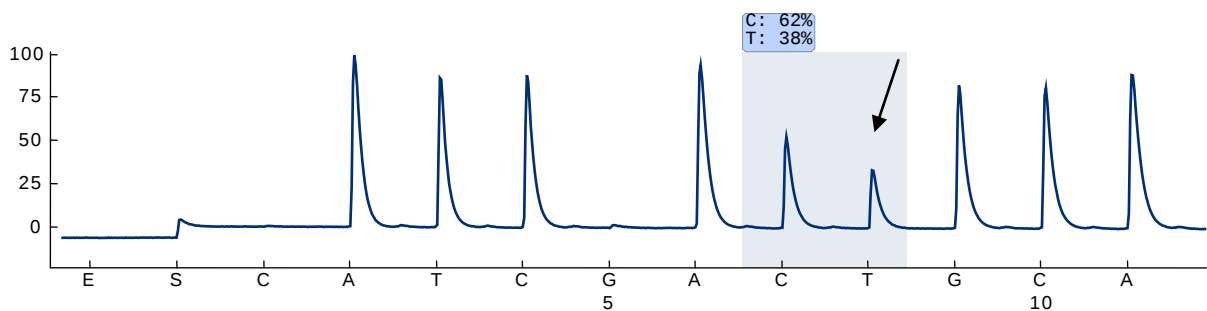


Figure 12. Pyrogram trace obtained after analysis of a sample with an ACG → ATG mutation in base 2 of codon 790 (indicated with an arrow).

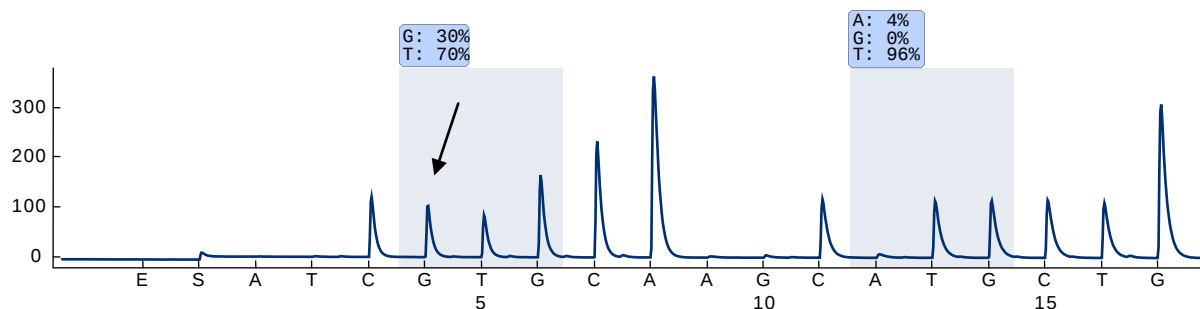


Figure 13. Pyrogram trace obtained after analysis of a sample with a CTG → CGG mutation in base 2 of codon 858 (indicated with an arrow).

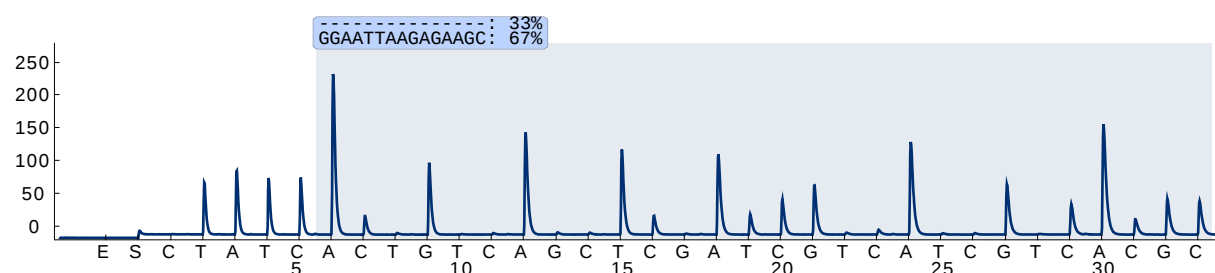


Figure 14. Pyrogram trace obtained after analysis of a sample with a 2235del15 deletion in exon 19.

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).

Note: 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. |

Comments and suggestions

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 (for example, the QIAxcel® System or agarose-gel electrophoresis). |
|----------------------------|--|

“Check” or “failed” result

- | | |
|---|---|
| a) Low peak height | <p>Handling errors in PCR setup or sample preparation prior to Pyrosequencing can result in low peaks. Perform the function test for filter probes as described in the <i>PyroMark Q24 User Manual</i> on a regular basis and exchange filter probes when indicated.</p> <p>In case of a “Check” warning carefully compare the Pyrogram to the histogram which can be displayed by a right click in the Pyrogram window. If the measured peaks match the height of the histogram bars, the result is valid. Otherwise, it is recommended to rerun the sample.</p> |
| b) Mutation not defined in “Sequence to Analyze” | Adjust the “Sequence to Analyze” in the assay setup (see Appendix A, page 51) 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 “Sequences to Analyze”. These samples should be analyzed using the alternative “Sequences to Analyze” considering unexpected mutations. |
| d) High peak height deviation warning at dispensation x | The Pyrogram should be carefully compared to the histogram, which can be displayed by a right click 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, it is recommended to rerun the sample. |

Comments and suggestions

- | | |
|---|---|
| d) Warning message
"Uncertain/Failed due to high peak height deviation at dispensation: 8" appears 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 Software). |
| e) Warning message
"Uncertain/Failed due to high peak height deviation at dispensation: 10." appears in the codon 858-861 assay. | <p>A high level CTG>CGG mutation in codon 858 (L858R) can result in increased background in dispensations G-10 and A-12 and a frequency above LOD for the mutation ATG>CAG in codon 861 (L861Q). In this case, only the reported L585R mutation is valid and the warning and "Check" quality can be disregarded.</p> <p>Note: The EGFR Plug-in Report will report only one mutation (i.e., the mutation with the highest frequency).</p> |
| f) Warning message
"Uncertain due to high peak height deviation at dispensation: 23." appears when the 2235del15 deletion is reported. | A high level 2235del15 deletion can result in this warning. In this case, the reported mutation is valid and the warning and "Check" quality can be disregarded. |

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. |

Comments and suggestions

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 <i>therascreen</i> reagents according to the instructions in "Protocol 5: Running the PyroMark Q24", page 26. |
| 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 *therascreen* EGFR Pyro Kit is tested against predetermined specifications to ensure consistent product quality.

Limitations

Any diagnostic results generated must be interpreted in conjunction with other clinical or laboratory findings.

It is the user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by the QIAGEN performance studies.

Performance Characteristics

Limit of blank and limit of detection

The limit of blank (LOB) and limit of detection (LOD) have been determined for a number of mutations using mixtures of plasmids (Table 9). LOB and LOD were determined according to the recommendations in the Clinical and Laboratory Standards Institute (CLSI) Guideline EP17-A "Protocol for determination of limits of detection and limits of quantitation; approved guideline". α - and β -errors (false positive and false negative, respectively) were set to 5%. LOD for some rare deletions in Exon 19 were determined by adding six standard deviations of blank measurements to the LOB value.

LOB values represent the measured frequency obtained with a wild-type sample. LOD values represent the lowest signal (measured frequency) that can be regarded as positive for the respective mutation.

The mutation CTG → CGG in codon 858

For the CTG → CGG mutation in codon 858, the measurements of samples with low levels of mutation had a non-Gaussian distribution. LOD was therefore determined using a different method, according to the recommendations in the CLSI Guideline EP17-A. The lowest signal that indicates the presence of a mutation (LOD) in these positions was set to 2 % units above the respective baseline level as defined by the 95th percentile of blank measurements. When analyzing a sample with a mutation level of 5.5%, 95% of results (n=72) gave a signal that can be regarded as positive ($\geq$ LOD, i.e., ≥ 2.6 % units).

Table 9. LOB and LOD determined for specific mutations

Mutation	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V47)
Exon 19 deletions				
2233del15	K745_E749del	0.6	1.6	26038
2235_2248>AATTC	E746_A750>IP	0.8	1.6	13550
2235_2252>AAT	E746_T751>I	1.1	2.8	13551
2235del15 [†]	E746_A750del	0.9	1.8	6223
2236del15 [†]	E746_A750del	0.2	1.2	6225
2237_2252>T	E746_T751>V	0.8	2.4	12386
2237_2255>T [†]	E746_S752>V	0.6	1.6	12384
2237del15 [†]	E746_T751>A	0.9	1.9	12678
2237del18	E746_S752>A	0.5	1.7	12367
2238_2248>GC	L747_A750>P	0.8	2.5	12422
2238_2252>GCA	L747_T751>Q	0.2	0.6	12419
2238del18	E746_S752>D	0.3	1.1	6220
2239_2248>C [†]	L747_A750>P	1.8	2.5	12382
2239_2251>C	L747_T751>P	0.6	1.7	12383
2239_2258>CA	L747_P753>Q	1.3	3.9	12387
2239del18 [†]	L747_S752del	0.6	1.5	6255
2239del9	L747_E749del	2.0	3.7	6218
2240del12	L747_T751>S	0.4	1.5	6210
2240del15 [†]	L747_T751del	0.9	1.9	12369
2240del18 [†]	L747_P753>S	0.9	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.

[†] The LOD for these deletions in Exon 19 were determined by adding six standard deviations of blank measurements to the LOB value.

Table continued on next page

Table 9. Continued

Mutation	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V47)
Exon 18 codon 719 (GGC)				
AGC	G719S	0.9	1.5	6252
TGC	G719C	1.0	1.6	6253
GCC	G719A	4.7	9.1	6239
Exon 20 codon 768 (AGC)				
ATC	S768I	2.6	5.0	6241
Exon 20 codon 790 (ACG)				
ATG	T790M	7.0	10.7	6240
Exon 21 codon 858 (CTG)				
CGG	L858R	0.6	2.6 (5.5) [‡]	6224
Exon 21 codon 861 (CTG)				
CAG	L861Q	3.2	4.3	6213
CGG	L861R	1.9	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.

Note: These values were based on runs where mixtures of plasmids carrying the wildtype or respective mutated sequence were used as template for PCR amplification.

Note: It is recommended that the method performance is confirmed in the laboratory.

Linearity

Linearity was determined using mixtures of plasmids carrying the wild-type or mutant sequence for the mutations GGC>AGC codon 719, ACG>ATG in codon 790, CTG>CGG in codon 858, and the deletions 2235del15 and 2236del15 in Exon 19. The plasmids were mixed in proportions to give four levels of mutation (5, 10, 30, and 50%). Each mixture was analyzed with three different lots of the *therascreen* EGFR Pyro Kit in three Pyrosequencing runs with three replicates each.

The results (n=9 for each mutation level) were analyzed according to the CLSI Guideline EP6-A “Evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline” using the Analyse-it® Software v2.21 and are shown in Figure 15 for the deletion 2235del15 in Exon 19.

The results were linear within an allowable nonlinearity of 5 % units in the tested range of 5 to 50% mutation level. Similar results were obtained for the mutations GGC>AGC in codon 719, ACG>ATG in codon 790, CTG>CGG in codon 858, and the deletion 2236del15 in Exon 19.

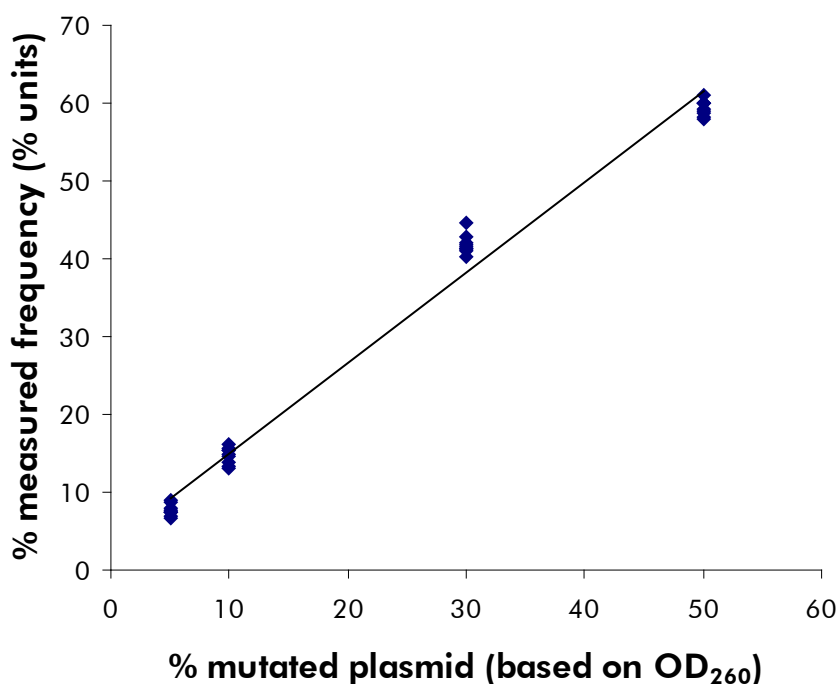


Figure 15. Linearity of deletion 2235del15 in Exon 19.

Precision

The precision data allows the determination of the total variability of the assays and was obtained at three different levels by analysis of the above mentioned plasmid mixtures with three replicates each.

Repeatability (intra-assay and inter-batch variability) was calculated based on the data for determination of linearity (three runs on the same day using varying lots of the *therascreen* EGFR Pyro Kit). Intermediate precision (intra-laboratory variability) was determined in three runs within one laboratory on three different days with varying operators, PyroMark Q24 instruments, and lots of the *therascreen* EGFR Pyro Kit. Reproducibility (inter-laboratory variability) was calculated from two runs each in an internal and external laboratory and using varying lots of the *therascreen* EGFR Pyro Kit.

Precision estimates are expressed as standard deviation of the measured mutation frequencies in % units (Table 10). The repeatability, intermediate precision, and reproducibility for the deletion 2235del15 in exon 19 was 0.8–1.2, 0.7–2.9, and 0.7–1.8 % units, respectively, in the measured range of 5 to 50% mutation level. Similar results were obtained for the mutations GGC>AGC in codon 719, ACG>ATG in codon 790, CTG>CGG in codon 858, and the deletion 2236del15 in exon 19.

Table 10. Precision for the deletion 2235del15 in Exon 19*

% mutated plasmid [†]	Repeatability		Intermediate precision		Reproducibility	
	Mean	SD	Mean	SD	Mean	SD
5	7.7	0.8	7.4	0.7	7.4	0.7
10	14.7	1.1	14.5	1.3	14.4	1.1
30	41.8	1.2	40.0	2.0	41.5	1.7
50	59.4	1.0	58.2	2.9	60.7	1.8

* All values are given as % units. SD: standard deviation (n=9).

[†] Based on OD₂₆₀ measurement.

Diagnostic evaluation

The *therascreen* EGFR Pyro Kit was evaluated in comparison with Sanger sequencing and the *therascreen* EGFR RGQ Kit. DNA was extracted from 100 formalin-fixed paraffin-embedded (FFPE) tumor samples from non-small cell lung cancer (NSCLC) and analyzed for mutations in codons 719, 768, 790, and 858–861, and deletions and complex mutations in exon 19.

DNA was isolated using the QIAamp DNA FFPE Tissue Kit. Analyses were carried out with the *therascreen* EGFR Pyro Kit on the PyroMark Q24, with the *therascreen* EGFR RGQ Kit on the Rotor Gene-Q 5plex HRM series II and Sanger sequencing was carried out on the ABI® 3130 Genetic Analyzer.

Of 100 samples analyzed the mutational status could be determined for all codons and exon 19 in 97 samples with all three methods. For two samples the mutational status of codon 768 could not be determined by Pyrosequencing, and one sample failed for most of the codons with all three methods, indicating the DNA quality was too low to allow amplification.

The T790M resistance mutation was detected in one sample with all three methods while the L861Q mutation was detected in one sample only by Pyrosequencing. Thirteen, twelve, and sixteen deletions and complex mutations in exon 19 were detected by Pyrosequencing, Rotor-Gene Q, and Sanger sequencing analysis, respectively. Three of the exon 19 deletions detected by Sanger sequencing could not be reproduced by both Pyrosequencing and Rotor-Gene Q analysis. The L858R mutation was detected in three samples by all three methods, in two samples by Pyrosequencing and either one of the other methods, in one sample by Pyrosequencing only, and in one sample by Rotor-Gene Q analysis only. The results are illustrated in Tables 11–14.

No mutations were detected in codons 719 and 768 in the 100 samples by any of the three methods.

Excluding samples that failed in one or more methods, the *therascreen* EGFR Pyro Kit and Sanger sequencing showed 100%, 98%, 99%, and 97% concordance in results for the codons 790, 858, 861, and exon 19, respectively, while the *therascreen* EGFR Pyro Kit and *therascreen* EGFR RGQ Kit showed 100%, 97%, 99%, and 99% concordance in results for the codons 790, 858, 861, and exon 19, respectively (Tables 11–14).

Table 11. Results of the analyzed NSCLC tumor samples for codon 790

		Sanger sequencing			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> EGFR Pyro Kit	Mutant	1	0	1	2
	Wild type	0	98	0	98
	Unknown	0	0	0	0
	Total	1	98	1	100
		<i>therascreen</i> EGFR RGQ Kit			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> EGFR Pyro Kit	Mutant	1	0	1	2
	Wild type	0	98	0	98
	Unknown	0	0	0	0
	Total	1	98	1	100

Table 12. Results of the analyzed NSCLC tumor samples for codon 858

		Sanger sequencing			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> EGFR Pyro Kit	Mutant	4	2	0	6
	Wild type	0	93	0	93
	Unknown	0	0	1	1
	Total	4	95	1	100
		<i>therascreen</i> EGFR RGQ Kit			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> EGFR Pyro Kit	Mutant	4	2	0	6
	Wild type	1	92	0	93
	Unknown	0	1	0	1
	Total	5	95	0	100

Table 13. Results of the analyzed NSCLC tumor samples for codon 861

		Sanger sequencing			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> EGFR Pyro Kit	Mutant	0	1	0	1
	Wild type	0	98	0	98
	Unknown	0	1	0	1
	Total	0	100	0	100
		<i>therascreen</i> EGFR RGQ Kit			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> EGFR Pyro Kit	Mutant	0	1	0	1
	Wild type	0	98	0	98
	Unknown	0	0	1	1
	Total	0	99	1	100

Table 14. Results of the analyzed NSCLC tumor samples for exon 19

		Sanger sequencing			
		Mutant	Wild type	Unknown	Total
therascreen EGFR Pyro Kit	Mutant	13	0	0	13
	Wild type	3	84	0	87
	Unknown	0	0	0	0
	Total	16	84	0	100
		therascreen EGFR RGQ Kit			
		Mutant	Wild type	Unknown	Total
therascreen EGFR Pyro Kit	Mutant	12	1	0	13
	Wild type	0	86	1	87
	Unknown	0	0	0	0
	Total	12	87	1	100

Note: In all runs used for determination of performance characteristics the signal was over 20 RLU for the codon 768 assay and over 30 RLU for the remaining four assays, as routinely obtained from 10 ng of DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue. The Pyrosequencing data was analyzed using the EGFR Plug-in Report.

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.

Symbols

 Σ $\langle N \rangle$ Contains reagents sufficient for $\langle N \rangle$ tests

 Use by

 IVD In vitro diagnostic medical device

 REF Catalog number

 LOT Lot number

 MAT Material number

 COMP Components

 CONT Contains

 NUM Number

 NaOH Sodium hydroxide

 GTIN Global Trade Item Number

 Temperature limitation

 Manufacturer

 Consult instructions for use

Contact Information

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).

Appendix A: Setting Up *therascreen* EGFR Pyro Assays

In case the EGFR Plug-in Report has been installed, predefined Assay Setups for codons 719, 768, 790, and 858–861, and exon 19 deletions are available in the shortcut browser of the PyroMark Q24 software under the path “Example Files/PyroMark Setups/EGFR”. The following steps do not need to be performed. The EGFR Plug-in Report can be obtained by e-mail from pyro.plugin@qiagen.com.

We strongly recommend the use of the EGFR Plug-in Report over manual analysis. Complex mutations cannot 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 *therascreen* EGFR Pyro assay for the first time. Set up the assay for EGFR codon 719, codon 768, codon 790, codons 858–861, and exon 19 deletions by using the PyroMark Q24 Software, as described below.

Procedure

EGFR codon 719

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

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

DGCTCCGGTGC

Note: 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 that 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

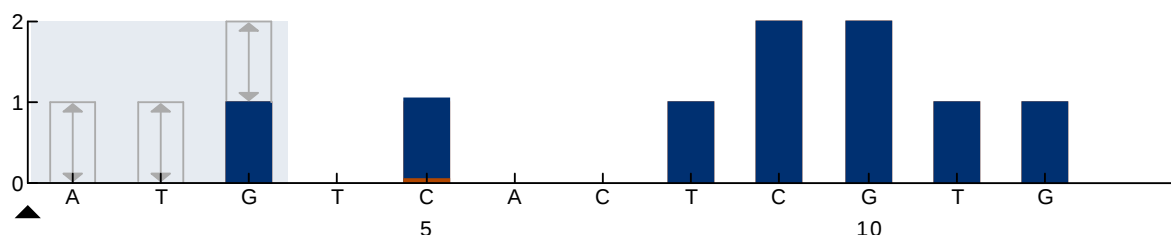


Figure 16. Histogram for codon 719 (nucleotide 2155) with the “Sequence to Analyze” DGCTCCGGTGC. The red rectangle at the bottom of the bar at dispensation C5 illustrates the adjustment of heights of histogram bar.

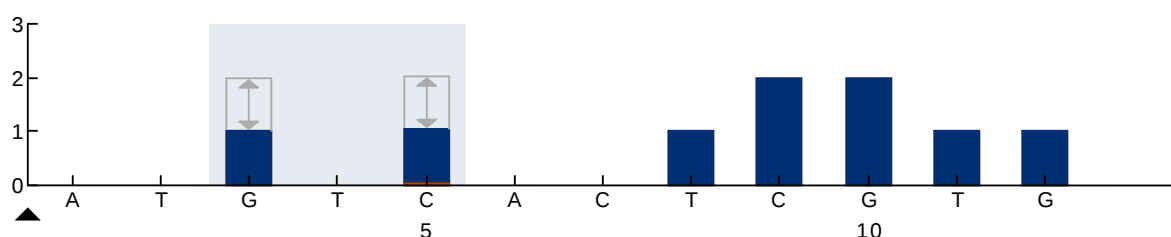


Figure 17. Histogram for codon 719 (nucleotide 2156) with the “Sequence to Analyze” GSCTCCGGTGC. The red rectangle at the bottom of the bar at dispensation C5 illustrates the adjustment of heights of histogram bar.

- 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

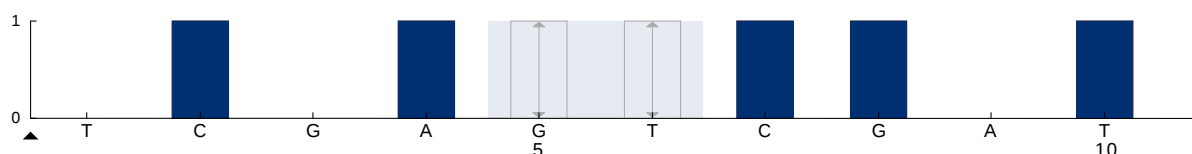


Figure 18. Histogram for codon 768 (nucleotide 2303) with the “Sequence to Analyze” CAKCGTG.

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

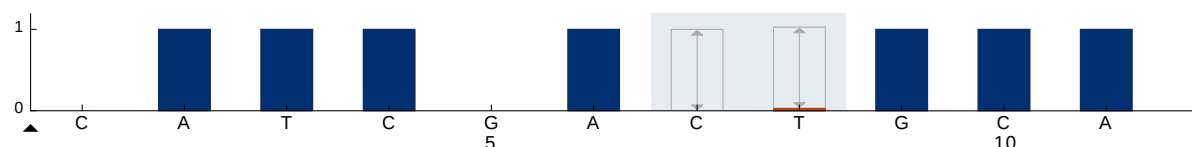


Figure 19. Histogram for codon 790 (nucleotide 2369) with the “Sequence to Analyze” ATCAYGCAG. The red rectangle at the bottom of the bar at dispensation T8 illustrates the adjustment of heights of histogram bar.

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 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

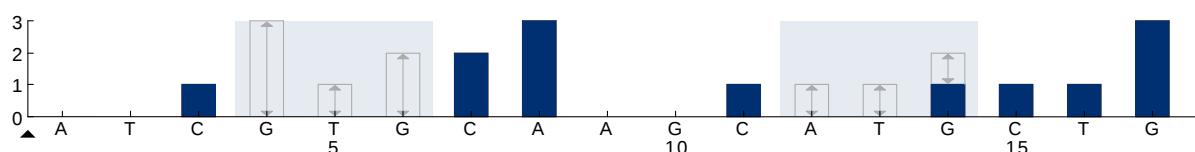


Figure 20. Histogram for codons 858–861 with the “Sequence to Analyze” CKGGCCAAACDGCTGGGT.

- 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 wild-type 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

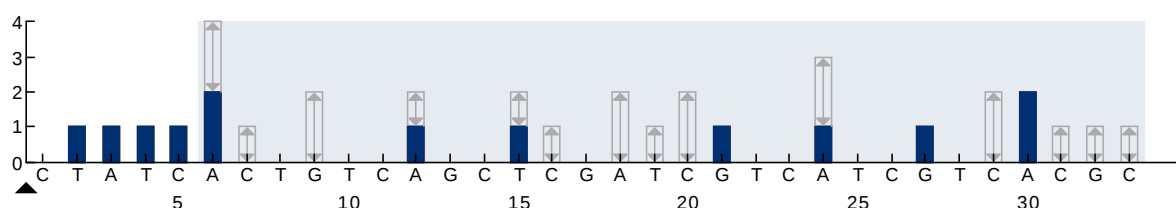


Figure 21. Histogram for exon 19 del.

- 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 Safety Data Sheets (SDSs) 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.
---	--

* 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.**
Note: 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*.**

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> 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	971480
PyroMark Q24 MDx	Sequence based detection platform for Pyrosequencing of 24 samples in parallel	9001513
PyroMark Q24	Sequence based detection platform for Pyrosequencing of 24 samples in parallel	9001514
PyroMark Q24 MDx Vacuum Workstation*	Vacuum Workstation (220 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001517* 9001515†
PyroMark Q24 Vacuum Workstation	Vacuum Workstation (220 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001518
PyroMark Q24 MDx Software	Application software	9019063
PyroMark Q24 Software	Analysis software	9019062
Accessories		
PyroMark Q24 Plate (100)	24-well sequencing reaction plate	979301
PyroMark Q24 Cartridge (3)	Cartridges for dispensing nucleotides and reagents	979302

* UK only.

† Rest of world.

Product	Contents	Cat. no.
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	979303
PyroMark Q24 Validation Oligo	For performance confirmation of system	979304
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

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

This page intentionally left blank

Trademarks: QIAGEN®, QIAamp®, QIAxcel®, BioRobot®, CorallLoad®, EZ1®, HotStarTaq®, MinElute®, Pyro®, Pyrogram®, PyroMark®, Pyrosequencing®, *therascreen*® (QIAGEN Group); ABI (Life Technologies Corporation); Analyse-it® (Analyse-it Software, Ltd.); Milli-Q® (Millipore Corporation); Sepharose® (GE Healthcare); Variomag (Florida Scientific Services, Inc.); Windows® (Microsoft Corporation).

Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the *therascreen* EGFR Pyro Kit to the following terms:

1. The *therascreen* EGFR Pyro Kit may be used solely in accordance with the *therascreen EGFR Pyro Kit Handbook* and for use with components contained in the kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this kit with any components not included within this kit except as described in the *therascreen EGFR Pyro Kit Handbook* and additional protocols available at www.qiagen.com.
2. Other than expressly stated licenses, QIAGEN makes no warranty that this kit and/or its use(s) do not infringe the rights of third-parties.
3. This kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the kit and/or its components.

For updated license terms, see www.qiagen.com.

© 2015 QIAGEN, all rights reserved.

www.qiagen.com

Australia ■ Orders 1-800-243-800 ■ Fax 03-9840-9888 ■ Technical 1-800-243-066

Austria ■ Orders 0800-28-10-10 ■ Fax 0800-28-10-19 ■ Technical 0800-28-10-11

Belgium ■ Orders 0800-79612 ■ Fax 0800-79611 ■ Technical 0800-79556

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)

