

therascreen[®] BRAF Pyro[®] Kit Handbook



Version 1



For in vitro diagnostic use



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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 reagents supplied with the <i>therascreen</i> BRAF 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	30
Interpretation of analysis results and detection of low level mutations	30
Troubleshooting guide	33
Quality Control	35
Limitations	35
Performance Characteristics	36
References	40
Symbols	41
Contact Information	41

Appendix A: Setting Up <i>therascreen</i> BRAF Pyro Assays	42
Appendix B: Emptying the Waste Container and Troughs	44
Ordering Information	45

Intended Use

The *therascreen* BRAF Pyro Kit is an *in vitro* nucleic acid sequence-based detection test based on Pyrosequencing[®] for the quantitative detection of mutations in codons 600 and 464–469 of the human BRAF gene in genomic DNA derived from human tissue samples.

The *therascreen* BRAF Pyro Kit is intended to provide clinicians with information to aid the selection of cancer patients more likely to benefit from 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* BRAF Pyro Kit is used for quantitative measurements of mutations in codons 600 and 464–469 of the human BRAF gene. The kit consists of two assays: one for detecting mutations in codon 600 and the other for detecting mutations in codons 464–469 (Figure 1). The two regions are amplified separately by PCR and sequenced through the defined region. Sequences surrounding the defined positions serve as normalization and reference peaks for quantification and quality assessment of the analysis.

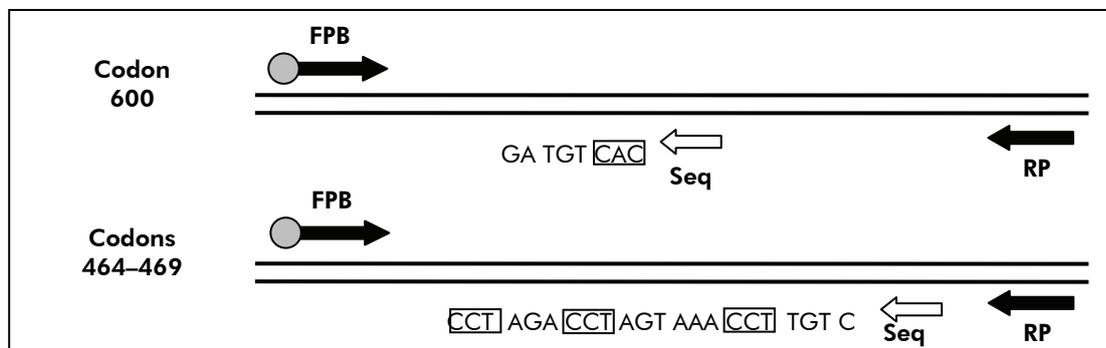


Figure 1. Illustration of the BRAF assay. The sequence indicated is the analyzed sequence for a wild-type sample. **FPB**: Forward PCR primers (B indicates biotinylation); **RP**: Reverse PCR primers; **Seq**: Sequencing primers.

Both assays are sequenced in the reverse direction.

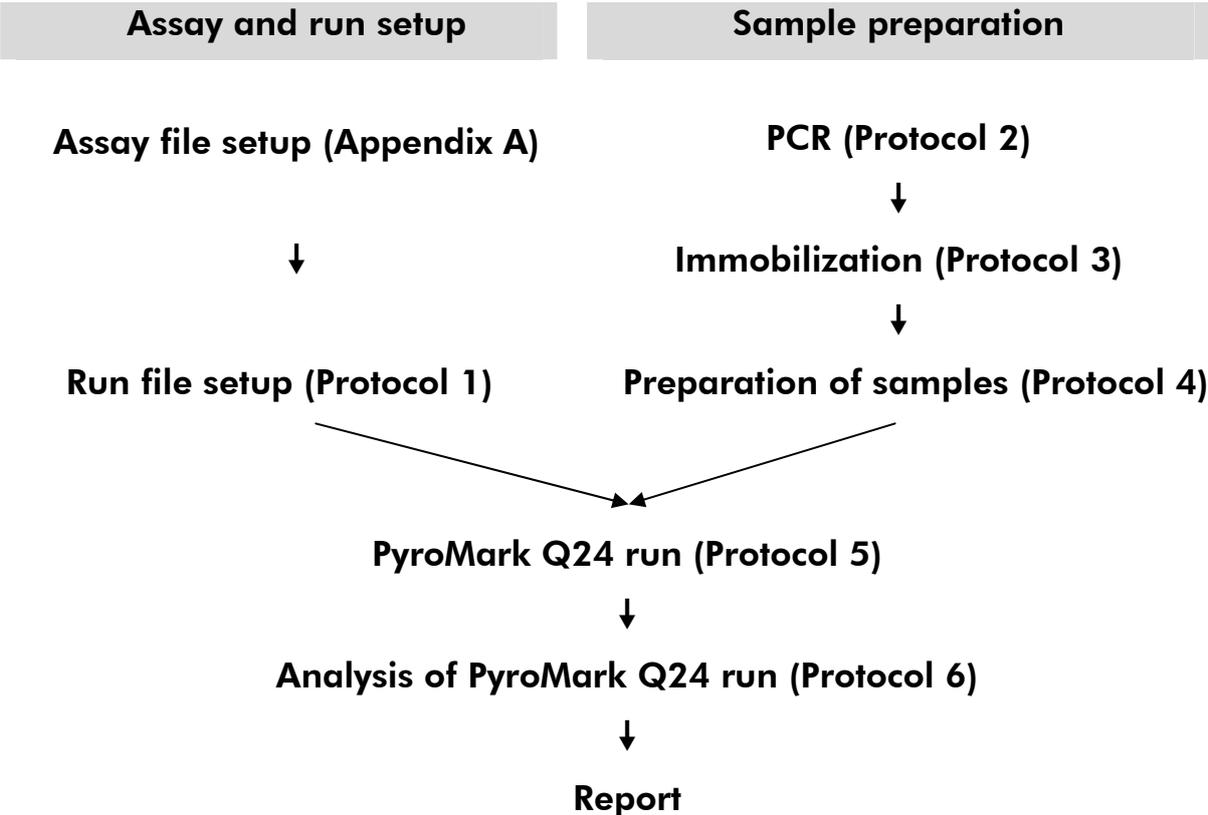
The product consists of a PCR primer mix and a sequencing primer for each assay. The primers are delivered in solution. Each vial contains 24 μ l of each primer or primer mix.

Principle of the Procedure

The workflow on page 6 illustrates the assay procedure. After PCR using primers targeting codon 600 and codons 464–469, the amplicons are immobilized on Streptavidin Sepharose® High Performance beads. Single-stranded DNA is prepared and the corresponding sequencing primers anneal to the DNA. The samples are then analyzed on the PyroMark Q24 using a run setup file and a run file. The “Sequence to Analyze” can be adjusted for detection of rare mutations after the run (see “Protocol 6: Analysis of a PyroMark Q24 run”, page 28).

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

Workflow of *therascreen BRAF 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 30). 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 BRAF Pyro Kit (box 1/2)

<i>therascreen</i> BRAF Pyro Kit (24)	(24)
Catalog no.	971470
Number of reactions	24
Seq Primer BRAF 600	24 μ l
Seq Primer BRAF 464–469	24 μ l
PCR Primer BRAF 600	24 μ l
PCR Primer BRAF 464–469	24 μ l
PyroMark PCR Master Mix, 2x	850 μ l
CoralLoad [®] Concentrate, 10x	1.2 ml
H ₂ O	3 x 1.9 ml
Unmethylated Control DNA, 10 ng/ μ l	100 μ l

therascreen buffers and reagents (box 2/2)

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

* Contains sodium hydroxide.

Materials Required but Not Provided

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), 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)*†
- 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, 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* BRAF Pyro Kit.

Table 1. Plate mixers recommended for use with the *therascreen* BRAF Pyro Kit

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

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 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.

The following risk and safety phrases apply to components of the *therascreen BRAF Pyro Kit*.

PyroMark Denaturation Solution

Contains sodium hydroxide: Irritant. Risk and safety phrases:* R36/38, S26-36/37/39-45

24-hour emergency information

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

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

General precautions

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 BRAF 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.

* R36/38: Irritating to eyes and skin; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice; S36: Wear suitable protective clothing; S37: Wear suitable gloves; S39: Wear eye/face protection; S45: In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

Reagent Storage and Handling

The *therascreen* BRAF Pyro Kit is shipped in two boxes. The *therascreen* BRAF Pyro Kit (box 1/2) is shipped on dry ice. PyroMark PCR Master Mix, CorallLoad Concentrate, Unmethylated Control DNA, and all primers should be stored at -15 to -25°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 -15 to -25°C . Frozen reagents should not be subjected to more than 3 freeze–thaw cycles.

Note: Nucleotides should not be frozen.

The *therascreen* BRAF 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 formalin-fixed paraffin-embedded (FFPE) samples.

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.

The QIAGEN[®] kits shown in Table 2 are recommended for DNA purification from the indicated human sample types for use with the *therascreen* BRAF 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* BRAF 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

* 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).

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

- Create an Assay Setup (see “Appendix A: Setting Up *therascreen* BRAF Pyro Assays”, page 42). This must only be done once before running the *therascreen* BRAF Pyro assay for the first time.

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 codon 600 and codons 464–469 to wells corresponding to the samples to analyze.

Note: A negative 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).

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 setup (see “Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads”, page 20).

To run the plate on PyroMark Q24 system, 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> BRAF 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 reagents supplied with the *therascreen* BRAF Pyro Kit

This protocol is for PCR amplification of a region containing codon 600 and a separate PCR amplification of a region containing codons 464–469 using the *therascreen* BRAF Pyro Kit.

Important points before starting

- The HotStarTaq[®] DNA polymerase in the PyroMark PCR Master Mix requires an activation step of **15 minutes at 95°C**.
- Set up all reaction mixtures in an area separate from that used for DNA purification, adding template 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 BRAF codon 600 or PCR Primer BRAF codons 464–469	1.0
Water (H ₂ O, supplied)	4.0
Total volume	20.0

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 (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. Capture 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 2 different sequencing primers in the same pattern as predefined for the plate in the run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 15), depending on the region of analysis (codon 600 or codons 464–469).
- The workflow has been slightly modified compared to revision R1 of the thescreen *BRAF 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 BRAF 600 or Seq Primer BRAF 464–469, 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 (μ l)	Volume for 9 + 1 reactions (μ l)
Seq Primer BRAF 600 or Seq Primer BRAF 464–469	0.8	8
PyroMark Annealing Buffer	24.2	242
Total volume	25	250

- 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 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.

- 3. 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. 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).**

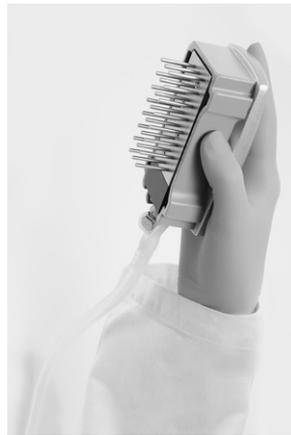


Figure 3. Illustration of the vacuum tool raised to beyond 90° vertical.

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 to not 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 vacuum tool (Off), and place the 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 44).
- 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 on how to set up a run, see the *PyroMark Q24 User Manual*.

Important point before starting

- The Pre Run information report, found in the “Tools” menu at run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 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: 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 material safety data sheets (MSDSs), available from the product supplier.

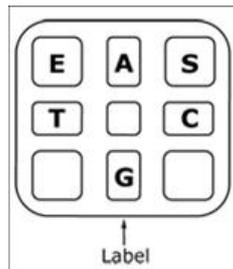


Figure 4. Illustration of the PyroMark Q24 Cartridge as seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (**E**), substrate mixture (**S**), and nucleotides (**A**, **T**, **C**, **G**) according to the volume information given in the Pre Run information 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 BRAF 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. To analyze the run and get an overview of the results click one of the Analyze buttons.



Analyze all wells.



Analyze the selected well.

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

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

Note: The most frequent mutation in BRAF is GTG → GAG at nucleotide 1799 (second base of codon 600). Therefore the standard "Sequence to Analyze" defined in the Analysis Setup addresses this mutation (see "Appendix A: Setting Up *therascreen* BRAF Pyro Assays", page 42). If a sample contains a mutation at nucleotide 1798 (first base of codon 600) or rare mutations at nucleotide 1799, the "Sequence to Analyze" can be changed to also analyze these mutations as described in Appendix A. Updated frequencies of mutations in the human BRAF gene in codon 600 and codons 464–469 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 30 RLU. Set 30 RLU as the "required peak height for passed quality" in assay setup (see Appendix A and the *PyroMark Q24 User Manual*).

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 GTG → GAG mutation detected 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 which received “Check” or “Failed” quality assessment. “Check” and “Failed” quality assessments may indicate a rare mutation that is not addressed by the standard “Sequence to Analyze” resulting in unexpected reference peaks.

To reanalyze and target mutations at nucleotide 1798, go to “Analysis Setup” and change “Sequence to Analyze” from CWCTGTAGC to CAYTGTAGC. Click “Apply” and then click “To All” when the “Apply Analysis Setup” window appears.

To reanalyze and target additional rare mutations at nucleotide 1799, go to “Analysis Setup” and change “Sequence to Analyze” to CVCTGTAGC. 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 is set to 30 RLU.

Note: If the measured peaks do not match the height of the histogram bars and cannot be explained by rare or unexpected mutations, 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, 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
- Mutation frequency $> \text{LOD} + 3\%$ units: Mutation

Note: 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.

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 BRAF mutation status.

Table 8. LOB and LOD determined for specific mutations

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V46)
Codon 600 (GTG), as assayed in reverse orientation (CAC)				
CTC	V600E	0.8	2.0	476
CCC	V600G	0.0	2.0 (5) [†]	6137
CGC	V600A	1.0	3.0 (7) [†]	18443
CAT	V600M	0.5	1.8	1130
Codon 464 (GGA), as assayed in reverse orientation (TCC)				
TTC	G464E	1.5	3.5 (8) [†]	449
Codon 466 (GGA), as assayed in reverse orientation (TCC)				
TAC	G466V	0.5	2.9	451
Codon 469 (GGA), as assayed in reverse orientation (TCC)				
TGC	G469A	1.4	3.8	460

* 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 5–7.

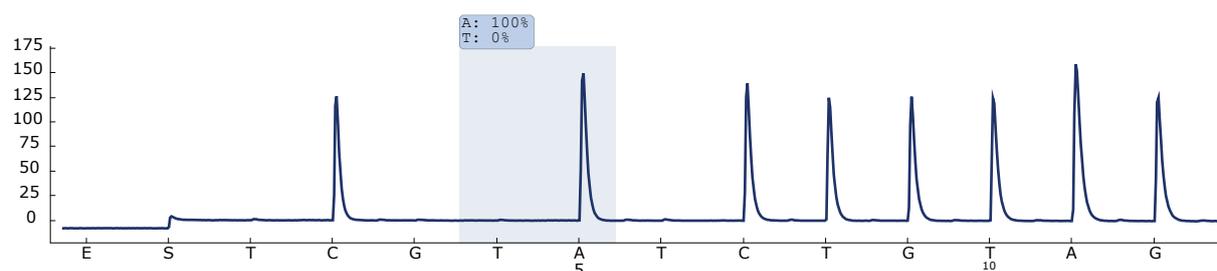


Figure 5. Pyrogram trace obtained after analysis of a sample with a wild type genotype in codon 600 with “Sequence to Analyze” CWCTGTAGC.

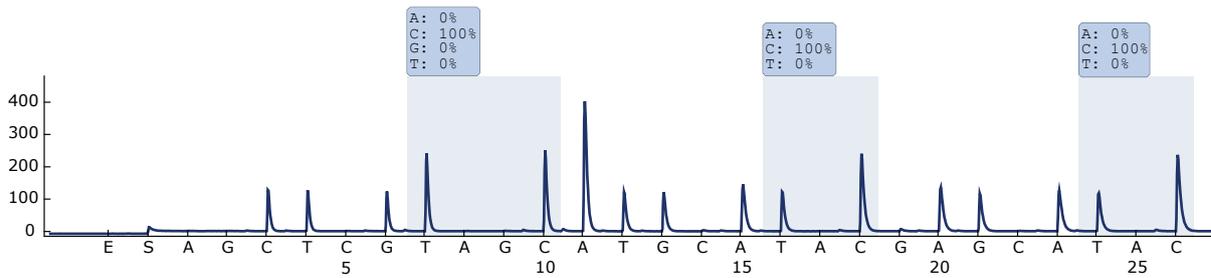


Figure 6. Pyrogram trace obtained after analysis of a sample with a wild type genotype in codons 464–469 with “Sequence to Analyze” CTGTTNCAAATGATHCAGATHCA.

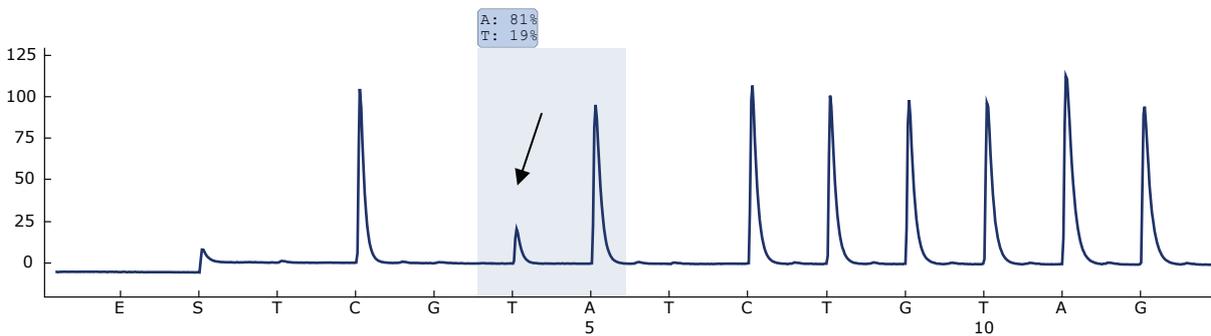


Figure 7. Pyrogram trace obtained after analysis of samples with a GTG → GAG (V600E) mutation in base 2 of codon 600 (nucleotide 1799, indicated with an arrow) with “Sequence to Analyze” CWCTGTAGC.

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. |

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 right-clicking 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> |
|--------------------|---|

Comments and suggestions

- b) Mutation not defined in "Sequence to Analyze" Adjust the sequence to analyze in the assay setup (see "Appendix A: Setting Up *therascreen* BRAF Pyro Assays", page 42), and reanalyze the run.
- c) Unexpected rare mutation A "Check" or "Failed" quality assessment can be caused by an unexpected pattern of peaks. This might indicate an unexpected mutation which is not analyzed by the provided "Sequence to Analyze". These samples should be analyzed using the alternative "Sequence to Analyze" considering unexpected mutations.
- d) High peak height deviation warning for a dispensation The Pyrogram should be carefully compared to the histogram, which can be displayed by 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.
- e) Warning message "The sequence contains less reference peaks than required" is appearing in the codon 600 assay with "Sequence to Analyze" CVCTGTAGC In case the measured peaks match the height of the histogram bars, the warning and the "Check" quality assessment can be disregarded.
- f) Warning message "High peak height deviation" for a dispensation in the variable position of the codon 600 assay with "Sequence to Analyze" CVCTGTAGC The run should be reanalyzed using the bi-allelic variants of the "Sequence to Analyze": CRCTGTAGC and CMCTGTAGC. Note that LOB and LOD values can differ for the bi-allelic variants.

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.

Comments and suggestions

- | | |
|---|---|
| b) Short cooling time of samples prior to Pyrosequencing analysis | Keep the samples on a PyroMark Q24 Plate Holder at room temperature for 10–15 minutes. Do not shorten the cooling time. |
| c) Contamination of cartridge | Carefully clean the cartridge as described in the product sheet. Store the cartridge protected from light and dust. |

No signals in positive controls (unmethylated control DNA)

- | | |
|---|---|
| a) Insufficient enzyme or substrate mix for all wells | Be 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 analysis 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 needed. Repeat the PCR and Pyrosequencing analysis. |

Quality Control

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

Limitations

Any diagnostic results that are 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". The α - and β -errors (false positive and false negative, respectively) were set to 5%. 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 mutations (GTG → GGG) and (GTG → GCG) in codon 600 and (GGA → GAA) in codon 464

For these mutations either blank measurements were consistently close to 0 % units (n=72) resulting in a non-Gaussian distribution or 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 the mutation level given in brackets in Table 9, 95% of results (n=72) gave a signal that can be regarded as positive (\geq LOD).

Table 9. LOB and LOD determined for specific mutations

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (V46)
Codon 600 (GTG), as assayed in reverse orientation (CAC)				
CTC	V600E	0.8	2.0	476
CCC	V600G	0.0	2.0 (5) [†]	6137
CGC	V600A	1.0	3.0 (7) [†]	18443
CAT	V600M	0.5	1.8	1130
Codon 464 (GGA), as assayed in reverse orientation (TCC)				
TTC	G464E	1.5	3.5 (8) [†]	449
Codon 466 (GGA), as assayed in reverse orientation (TCC)				
TAC	G466V	0.5	2.9	451
Codon 469 (GGA), as assayed in reverse orientation (TCC)				
TGC	G469A	1.4	3.8	460

* 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 are based on runs where mixtures of plasmids carrying the wild type 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 mutation V600E (GTG → GAG) in codon 600 of the BRAF gene. 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* BRAF 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 8 for the mutation V600E (GTG → GAG) in codon 600.

The results were linear within an allowable nonlinearity of 5 % units in the tested range of 5 to 50% mutation level.

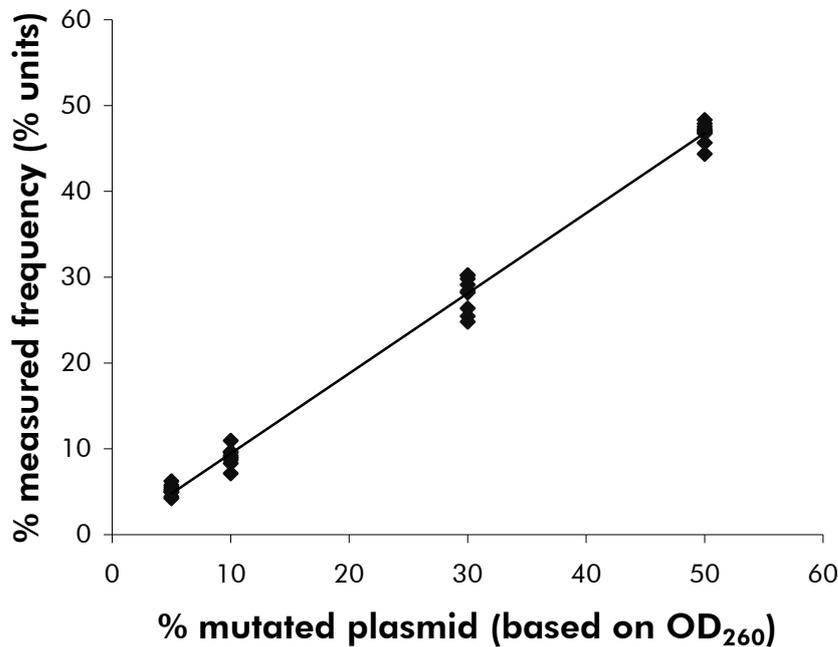


Figure 8. Linearity for the mutation V600E (GTG → GAG) in codon 600.

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* BRAF Pyro Kit). Intermediate precision (intra-laboratory variability) was determined in three runs within one laboratory on three different days with varying operators, PyroMark Q24 systems, and lots of the *therascreen* BRAF 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* BRAF 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 mutation V600E (GTG → GAG) in codon 600 was 0.6–2.1, 0.7–1.8, and 0.8–2.1 % units, respectively, in the measured range of 5 to 50% mutation level.

Table 10. Precision for the mutation V600E (GTG → GAG) in codon 600*

% mutated plasmid [†]	Repeatability		Intermediate precision		Reproducibility	
	Mean	SD	Mean	SD	Mean	SD
5	5.2	0.6	4.4	0.7	5.1	0.8
10	9.1	1.0	9.6	1.0	9.6	1.3
30	28.1	2.1	27.9	1.8	28.3	2.1
50	46.9	1.2	46.3	1.5	47.9	1.7

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

[†] Based on OD₂₆₀ measurement.

Diagnostic evaluation

The *therascreen* BRAF Pyro Kit was evaluated in comparison with Sanger sequencing. DNA was extracted from 100 formalin-fixed paraffin-embedded (FFPE) tumor samples from skin and analyzed for mutations in codon 600 and codons 464–469.

DNA was isolated using the QIAamp DNA FFPE Tissue Kit. Pyrosequencing analysis was carried out with the *therascreen* BRAF Pyro Kit on the PyroMark Q24 system and Sanger sequencing on the ABI™ 3130 Genetic Analyzer.

Of 100 samples analyzed, the mutational status of codon 600 and codons 464–469 could be determined in all and 99 samples with Sanger sequencing and the *therascreen* BRAF Pyro Kit, respectively (Table 11).

In four of the 100 samples, a V600E (GTG → GAG) mutation was detected by Sanger sequencing. Three of those samples had identical results with the *therascreen* BRAF Pyro Kit, while one sample failed in Pyrosequencing analysis for codon 600 due to low peaks. In the codons 464–469 assay, this sample had sufficient but considerably lower peaks than other samples indicating that the DNA was of low quality. None of the rare mutations in codons 464–469 were detected by both methods.

Excluding the sample that failed in one method, the *therascreen* BRAF Pyro Kit and Sanger sequencing showed 100% concordance in results for both the codon 600 and codons 464–469 (Tables 11 and 12).

Table 11. Results of the analyzed skin tumor samples for codon 600

		Sanger sequencing			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> BRAF Pyro Kit	Mutant	3	0	0	3
	Wild type	0	96	0	96
	Unknown	1	0	0	1
	Total	4	96	0	100

Table 12. Results of the analyzed skin tumor samples for codons 464–469

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

Note: In all runs used for determination of performance characteristics, the signal was over 30 RLU as routinely obtained from 10 ng of DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue.

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

	Contains reagents sufficient for <N> tests
	Use by
	In vitro diagnostic medical device
	Catalog number
	Lot number
	Material number
	Components
	Contains
	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* BRAF Pyro Assays

Before running the *therascreen* BRAF Pyro assay for the first time, the assay file must be set up. Set up the assay for BRAF codon 600 and codons 464–469 by using the PyroMark Q24 Software, as described below.

Procedure

BRAF codon 600

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

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

CWCTGTAGC

Note: The most frequent mutation in codon 600 is a GTG → GAG in nucleotide 1799 (second position).

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

To check if mutations are present in nucleotide 1798 (first position), change the “Sequence to Analyze” to the following sequence.

CAYTGTAGC

For additional rare mutations in nucleotide 1799, the “Sequence to Analyze” **CVCTGTAGC** should also be analyzed.

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

A3. Manually enter the following “Dispensation Order”.

TCGTATCTGTAG

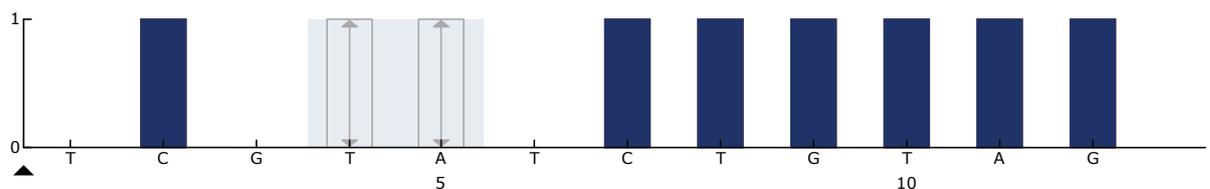


Figure 9. Histogram for codon 600 (nucleotide 1799) with the “Sequence to Analyze” CWCTGTAGC.

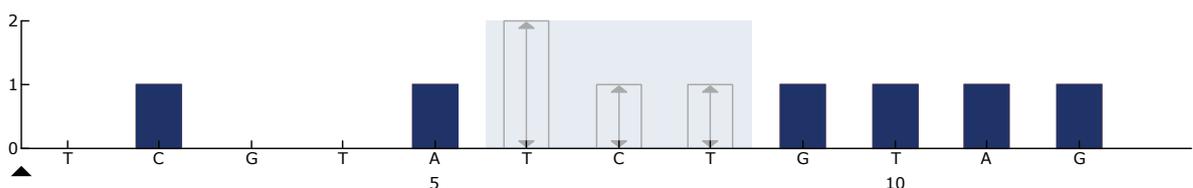


Figure 10. Histogram for codon 600 (nucleotide 1798) with the “Sequence to Analyze” CAYTGTAGC.

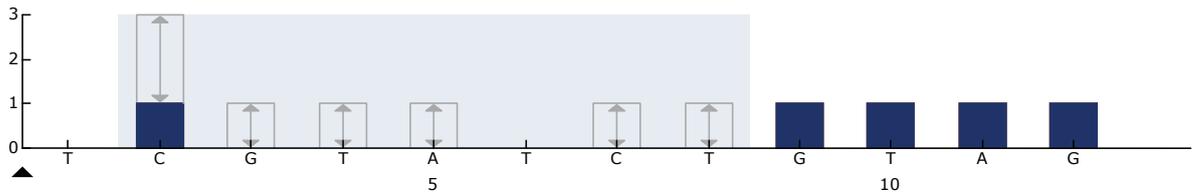


Figure 11. Histogram for codon 600 (nucleotide 1799) with the "Sequence to Analyze" CVCTGTAGC.

- 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 then save the assay as "BRAFcodon 600".

BRAF codons 464–469

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

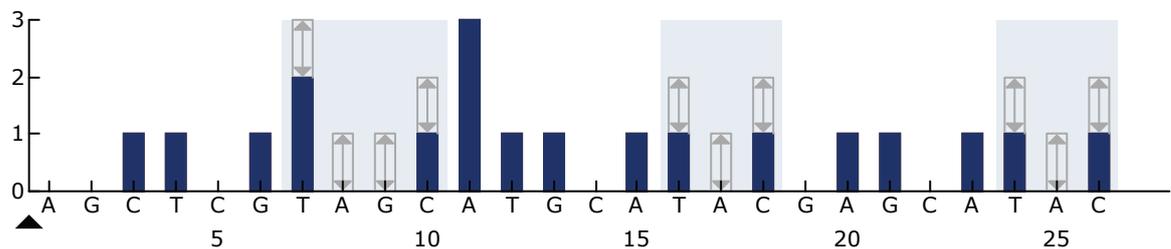


Figure 12. Histogram for codons 464–469 (nucleotides 1391 [codon 464], 1397 [codon 466], and 1406 [codon 469]).

- 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 "BRAFcodons 464–469".

Appendix B: Emptying the Waste Container and Troughs

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

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

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

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

Important point before starting

- This protocol requires high-purity water.

Procedure

- B1. Ensure that no vacuum is applied to the vacuum tool. Make sure that the vacuum is closed (Off) and the vacuum pump is switched off.**
- B2. Discard any solutions left in the troughs.**
- B3. Rinse the troughs with high-purity water or replace them, if necessary.**
- B4. Empty the waste container.**

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> BRAF 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	971470
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

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.

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