

March 2015

# *therascreen*<sup>®</sup> KRAS Pyro<sup>®</sup> Kit Handbook



Version 1



For in vitro diagnostic use



971460



1061825EN



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GERMANY

R3



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Sample & Assay Technologies

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## Intended Use

The *therascreen* KRAS Pyro Kit is an *in vitro* nucleic acid sequence-based detection test based on Pyrosequencing® technology, for the quantitative detection of mutations in codons 12, 13, and 61 of the human KRAS gene in genomic DNA derived from human tissue samples.

The *therascreen* KRAS Pyro Kit is intended to be used as an aid to identify colorectal cancer patients more likely to benefit from anti-EGFR therapies, such as panitumumab and cetuximab. For *in vitro* diagnostic use.

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

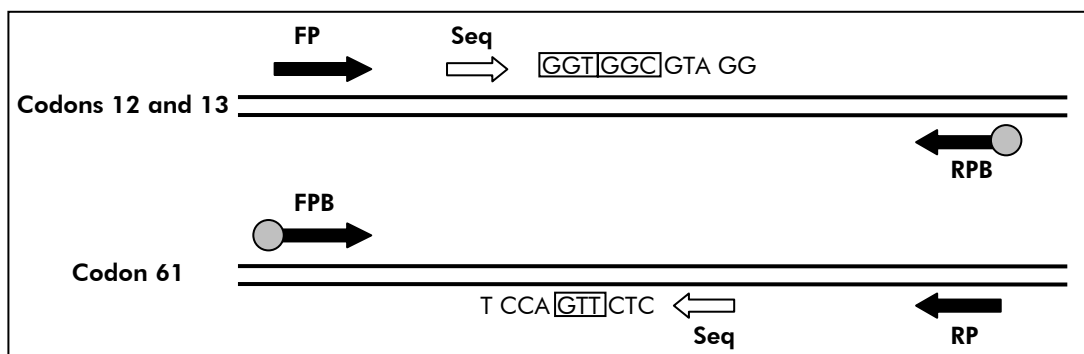
- The PyroMark Q24 instrument and the PyroMark Q24 MDx instrument.
- The PyroMark Q24 Vacuum Workstation and the PyroMark Q24 MDx Vacuum Workstation.
- PyroMark Q24 Software (version 2.0) and PyroMark Q24 MDx Software (version 2.0).

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

There is a strong focus on KRAS mutation analysis in Europe due to the European Commission's granting of conditional marketing authorization for panitumumab and cetuximab for treatment of metastasized colon cancer for patients with nonmutated (wild-type) KRAS gene. This means that panitumumab and cetuximab can only be given to patients who have been screened for KRAS mutation status.

The CE-IVD marked *therascreen* KRAS Pyro Kit is for quantitative measurements of mutations in codons 12, 13, and 61 of the human KRAS gene. The product consists of two assays: one for detecting mutations in codons 12 and 13 and the second for detecting mutations in codon 61 (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 KRAS assay.** The sequence indicated is the analyzed sequence for a wild-type sample. **FP** and **FPB**: Forward PCR primers (B indicates biotinylation); **RP** and **RPB**: Reverse PCR primers (B indicates biotinylation); **Seq**: Sequencing primers.

**Note:** Codons 12 and 13 are sequenced in the forward direction and codon 61 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  $\mu$ l of each primer or primer mix.

## Principle of the Procedure

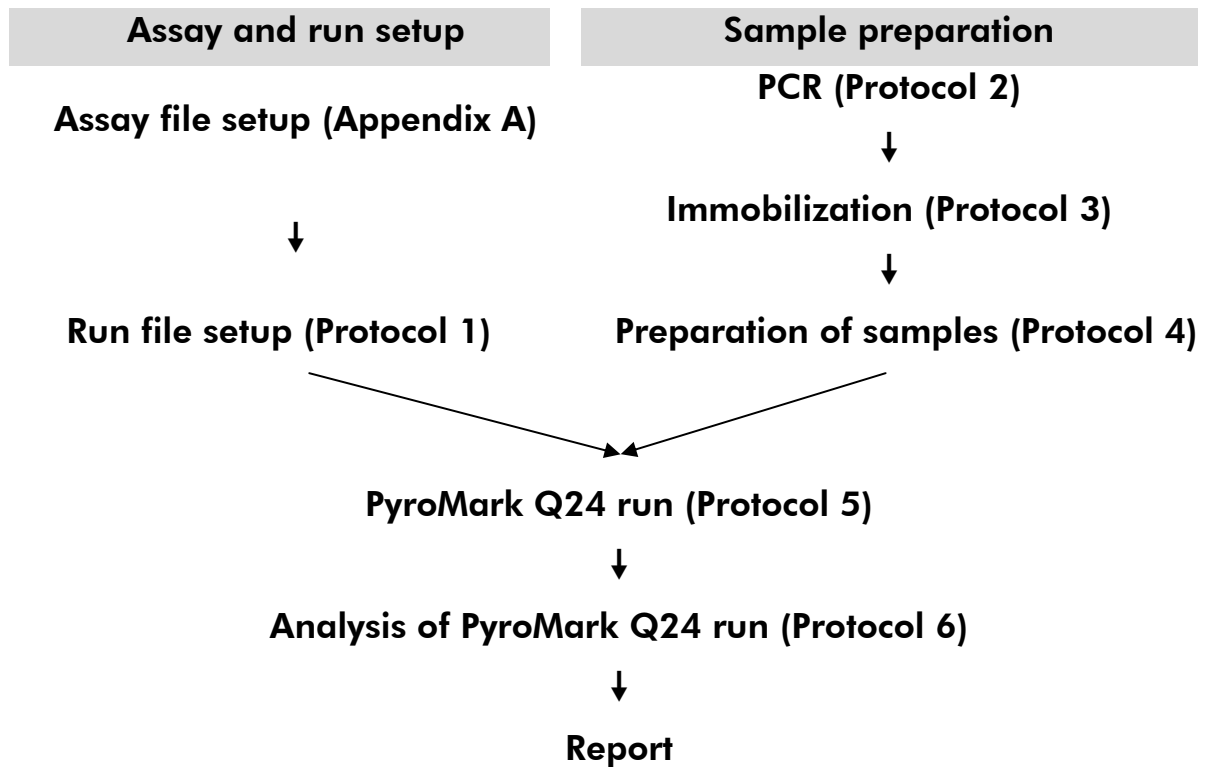
The workflow illustrates the assay procedure. After PCR using primers targeting codons 12/13 and codon 61, the amplicons are immobilized on Streptavidin Sepharose<sup>®</sup> High Performance beads. Single-stranded DNA is prepared and the corresponding sequencing primers anneal to the DNA. The samples are then analyzed on the PyroMark Q24 system using a run setup file and a run file.

It is recommended to use the KRAS Plug-in Report to analyze the run. The KRAS Plug-in Report can be obtained by e-mail from [pyro.plugin@qiagen.com](mailto: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 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 the *PyroMark KRAS Kit Handbook* and revision R1 of the *therascreen KRAS Pyro Kit Handbook* (see "Protocol 2: PCR using the PCR reagents supplied with the *therascreen KRAS Pyro Kit*", page 17, and "Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24", page 22).

## Workflow of *therascreen* KRAS Pyro procedure



### Controls

Unmethylated control DNA is included in the kit as a positive control for PCR and sequencing reactions. This control sample 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* KRAS Pyro Kit (box 1/2)

<b><i>therascreen</i> KRAS Pyro Kit</b>	<b>(24)</b>
<b>Catalog no.</b>	<b>971460</b>
<b>Number of reactions</b>	<b>24</b>
Seq Primer KRAS 12/13	24 $\mu$ l
Seq Primer KRAS 61	24 $\mu$ l
PCR Primer KRAS 12/13	24 $\mu$ l
PCR Primer KRAS 61	24 $\mu$ l
PyroMark PCR Master Mix, 2x	850 $\mu$ l
CoralLoad <sup>®</sup> Concentrate, 10x	1.2 ml
H <sub>2</sub> O	3 x 1.9 ml
Unmethylated Control DNA, 10 ng/ $\mu$ l	100 $\mu$ l



## **therascreen buffers and reagents (box 2/2)**

<b>therascreen buffers and reagents</b>		
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 $\alpha$ S		1180 $\mu$ l
dCTP		1180 $\mu$ l
dGTP		1180 $\mu$ l
dTTP		1180 $\mu$ 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](http://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* KRAS Pyro Kit.

**Table 1. Plate mixers recommended for use with the *therascreen* KRAS 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 <sup>®</sup> 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](http://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* KRAS 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 2: PCR using the PCR reagents supplied with the *therascreen* KRAS Pyro Kit”, page 17, and “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 22) compared to the *PyroMark* KRAS Kit Handbook and revision R1 of the *therascreen* KRAS Pyro Kit Handbook.
- The components of this product are sufficient to perform the 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* KRAS Pyro Kit is shipped in two boxes. The *therascreen* KRAS 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 *therascreen* buffers, enzyme mixture, substrate mixture, dATP $\alpha$ 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* KRAS 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<sup>®</sup> DNA Tissue Kit and the QIAamp<sup>®</sup> 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<sup>®</sup> kits shown in Table 2 are recommended for DNA purification from the indicated human sample types for use with the *therascreen* KRAS 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* KRAS Pyro Kit**

<b>Sample material</b>	<b>Nucleic acid isolation kit</b>	<b>Catalog number (QIAGEN)</b>
Paraffin-embedded tissue	QIAamp DNA FFPE Tissue Kit (50)	56404
	EZ1 DNA Tissue Kit (48)*	953034
Blood	QIAamp DSP DNA Blood Mini Kit <sup>†</sup>	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<sup>®</sup> EZ1 (cat. no. 9000705; no longer available) and the EZ1 DNA Paraffin Section Card (cat. no. 9015862).

<sup>†</sup> 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 KRAS Plug-in Report has not been installed, create an Assay Setup (see Appendix A, page 44). This must only be done once before running the *therascreen* KRAS Pyro assays for the first time. In case the KRAS Plug-in Report has been installed, pre-defined Assay Setups are available in the shortcut browser of the PyroMark Q24 software under the path “Example Files/PyroMark Setups/KRAS”. The KRAS Plug-in Report can be obtained by e-mail from [pyro.plugin@qiagen.com](mailto: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 both codons 12/13 and codon 61 to wells corresponding to the samples to analyze.**

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

**Note:** Include a sample with unmethylated control DNA for each assay in every Pyrosequencing run (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:	<b>Optional:</b> Enter ID of the PyroMark Q24 Plate.
Bar code:	<b>Optional:</b> 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 ID:	<b>Optional:</b> Enter the lot number for the <i>therascreen</i> KRAS Pyro Kit to be used. The lot number can be found on the product label. <b>Note:</b> We recommend entering both the reagent ID and the kit ID so that any unexpected problems with the reagents can be traced.
Run note:	<b>Optional:</b> 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* KRAS Pyro Kit

This protocol is for PCR amplifications of a region containing codon 12 and codon 13 and a separate PCR amplification of a region containing codon 61 using the *therascreen* KRAS Pyro Kit.

### Important points before starting

- The workflow has been slightly modified compared to the *PyroMark* KRAS Kit Handbook (step 5).
- The HotStarTaq<sup>®</sup> 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/ $\mu$ l, if necessary.

### Procedure

- 1. Thaw all necessary reagents (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**

<b>Component</b>	<b>Volume/reaction (<math>\mu</math>l)</b>
PyroMark PCR Master Mix, 2x	12.5
CoralLoad Concentrate, 10x	2.5
PCR Primer KRAS 12/13 <b>or</b> PCR Primer KRAS 61	1.0
Water (H <sub>2</sub> O, supplied)	4.0
<b>Total volume</b>	<b>20.0</b>

**3. Mix the reaction mix thoroughly and dispense 20  $\mu$ 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  $\mu$ 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**

<b>Component</b>	<b>Volume/reaction (<math>\mu</math>l)</b>
Reaction mix	20
Sample DNA	5
<b>Total volume</b>	<b>25</b>

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

**Table 5. Optimized cycling protocol**

			Comments
<b>Initial activation step:</b>	15 minutes	95°C	HotStarTaq DNA polymerase is activated by this heating step.
<b>3-step cycling:</b>			
Denaturation	20 seconds	95°C	
Annealing	30 seconds	53°C	
Extension	20 seconds	72°C	
Number of cycles	42		
<b>Final extension:</b>	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 <sub>2</sub> O, supplied)	28
<b>Total volume</b>	<b>70</b>

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 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 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 (codons 12 and 13, or codon 61)
- The workflow has been slightly modified compared to revision R1 of the theascreen *KRAS 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 KRAS 12/13 and Seq Primer KRAS 61, 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**

<b>Component</b>	<b>Volume/sample (<math>\mu</math>l)</b>	<b>Volume for 9 + 1 reactions (<math>\mu</math>l)</b>
Seq Primer KRAS 12/13 <b>or</b> Seq Primer KRAS 61	0.8	8
PyroMark Annealing Buffer	24.2	242
<b>Total volume</b>	<b>25</b>	<b>250</b>

- 2. Add 25  $\mu$ 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.**

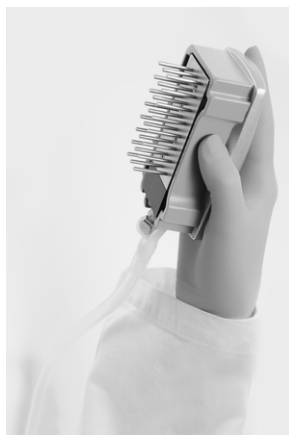
- 4. Apply vacuum to the tool by opening the vacuum switch.**
- 5. Carefully lower the filter probes of the vacuum tool into the PCR plate (or strips) to capture the beads containing immobilized**

**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 the tool 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 47).
- 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  $\mu$ l each of water ( $H_2O$ , supplied).**
2. **Mix by swirling the vial gently.**

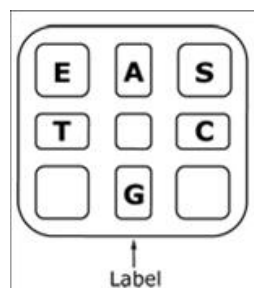
**Note:** Do not vortex!

**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 KRAS 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 KRAS 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 KRAS Plug-in Report for result interpretation. The KRAS Plug-in Report can be obtained by e-mail from [pyro.plugin@qiagen.com](mailto:pyro.plugin@qiagen.com). This report ensures that the respective LOD values (Table 8) and different "Sequences to Analyze" are used to automatically detect all mutations.

### 5. Using the KRAS Plug-in Report:

To generate a report, select "AQ Add On Reports/KRAS" and "Codon 12 and 13" or "Codon 61" from "Reports" in the menu.

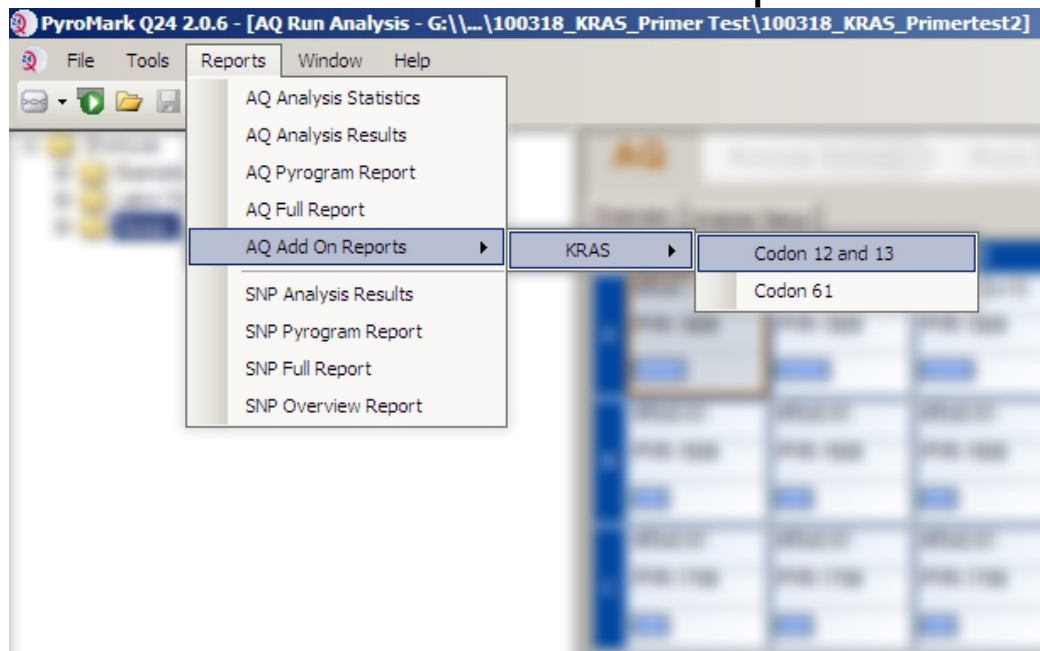


Figure 5. The AQ Run Analysis screen.

The wells will automatically be analyzed for all mutations for which 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

NOTE: Only the mutation with the highest frequency is reported.

Well	Sample ID	Result	Frequency [% units]	Nucleotide Substitution	Amino Acid Substitution	Info
A1	106506B1	Mutation	28.8	GGT>AGT	G12S	
A2	1090814B	Wildtype				
A3	110456B2	Potential low level mutation	2.3	GGT>AGT	G12S	⚠
A4	110457B2	Wildtype				
A5	110462A2	Wildtype				
A6	110486A2	Mutation	24.9	GGT>GCT	G12A	
A7	111207A2	Mutation	31.6	GGT>GTT	G12V	
A8	111555A2	Mutation	39.7	GGT>GAT	G12D	
B1	111565A2	Mutation	37.5	GGT>GAT	G12D	
B2	111667A2	Mutation	26.7	GGT>GTT	G12V	
B3	111670A2	Wildtype				

⚠ See detailed results for further explanation.

Figure 6. The results summary table.

## 6. Using AQ analysis:

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<sup>®</sup> 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 most frequent mutations in KRAS are found at nucleotide 35 (second base of codon 12). Therefore, the standard “Sequence to Analyze” for the KRAS Codon 12 and 13 assay as defined in the Analysis Setup addresses mutations at this position (see Appendix A, page 44). If a sample contains a mutation at nucleotide 34 (first base of codon 12), the “Sequence to Analyze” can be changed to analyze also the mutation status at this position, as described in Appendix A. Similarly, the “Sequence to Analyze” can be changed for the KRAS Codon 61 assay, as described in Appendix A.

Updated frequencies of mutations in the human KRAS gene in codon 12/13 and codon 61 are provided online by the Sanger Institute at [www.sanger.ac.uk/genetics/CGP/cosmic/](http://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 mutation detected in nucleotide 35 (Codon 12) or 183 (Codon 61), 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 a “Check” or “Failed” quality assessment. “Check” and “Failed” quality assessments may indicate a mutation in a position other than nucleotide 35 or 183, resulting in peak height deviations at reference dispensations. For example, a peak in any of the first 3 dispensations shows that a mutation is present at nucleotide 34.

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

To reanalyze and target mutations at nucleotide 182 (second position of Codon 61), change the “Sequence to Analyze” of the Codon 61 assay to the following sequence.

*CTCTHGACCTG*

To reanalyze and target mutations at nucleotide 181 (first position of Codon 61), change the “Sequence to Analyze” of the Codon 61 assay to the following sequence.

*CTCTTSACCTG*

**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, 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 the 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  $<$  LOD: Wild type
- Mutation frequency  $\geq$  LOD and  $\leq$  LOD + 3 % units: Potential low-level mutation

**Note:** If using the Plug-in Report (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  $\geq$  LOD and different from the control sample. Otherwise, the sample should be judged as wild type.

- Mutation frequency  $>$  LOD + 3 % units: Mutation

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

**Note:** It is recommended to use the KRAS 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 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 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:** The KRAS Plug-in Report algorithm was used to generate the LOB and LOD data. Manual analysis using the PyroMark Application Software as described in Protocol 6 (page 28) may result in slightly different values.

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

**Table 8. LOB and LOD determined for specific mutations**

<b>Nucleic acid substitution</b>	<b>Amino acid substitution</b>	<b>LOB (% units)</b>	<b>LOD (% units)</b>	<b>COSMIC ID* (V42)</b>
<b>Codon 12 (GGT)</b>				
GAT	G12D	0.6	2.2	521
GTT	G12V	0.0	1.0 (7) <sup>†</sup>	520
TGT	G12C	0.5	2.1	516
AGT	G12S	0.4	1.9	517
GCT	G12A	0.7	2.3	522
CGT	G12R	0.3	1.8	518
<b>Codon 13 (GGC)</b>				
GAC	G13D	0.3	1.9	532
<b>Codon 61 (CAA), as assayed in reverse orientation (TTG)</b>				
GTG	Q61H	0.8	2.8	554
TAG	Q61L	1.2	3.1	553
TCG	Q61R	1.6	3.5	552
ATG	Q61H	0.7	2.6	555
TTC	Q61E	1.2	3.1	550

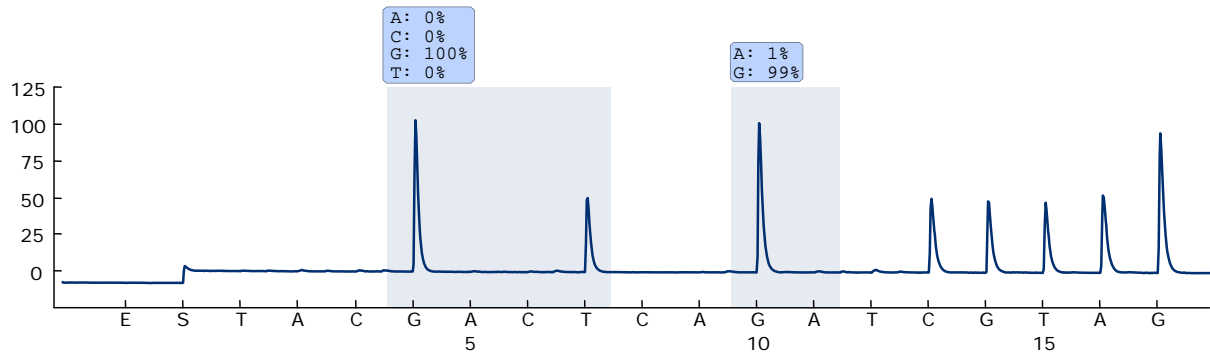
\* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at [www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/).

<sup>†</sup> Lowest mutation level in a sample resulting in a measured frequency  $\geq$ LOD.

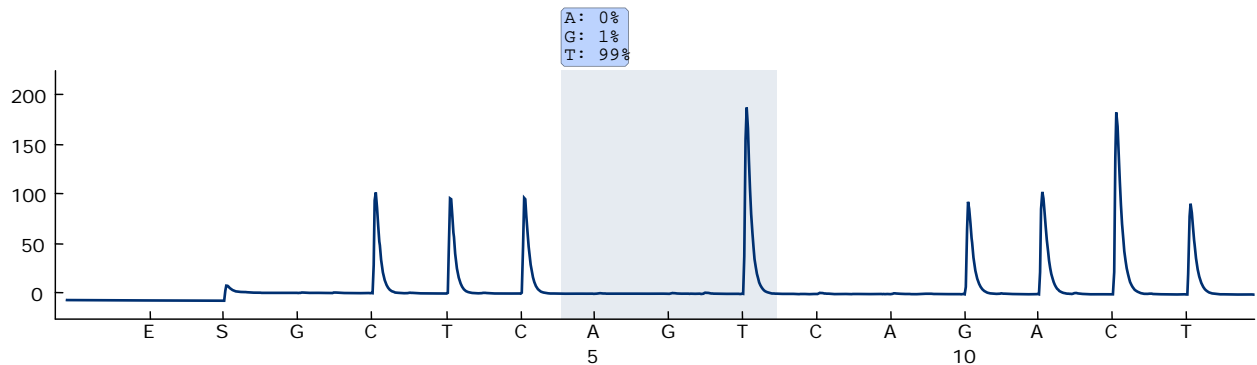


## Representative results using the AQ analysis integral to the PyroMark Q24

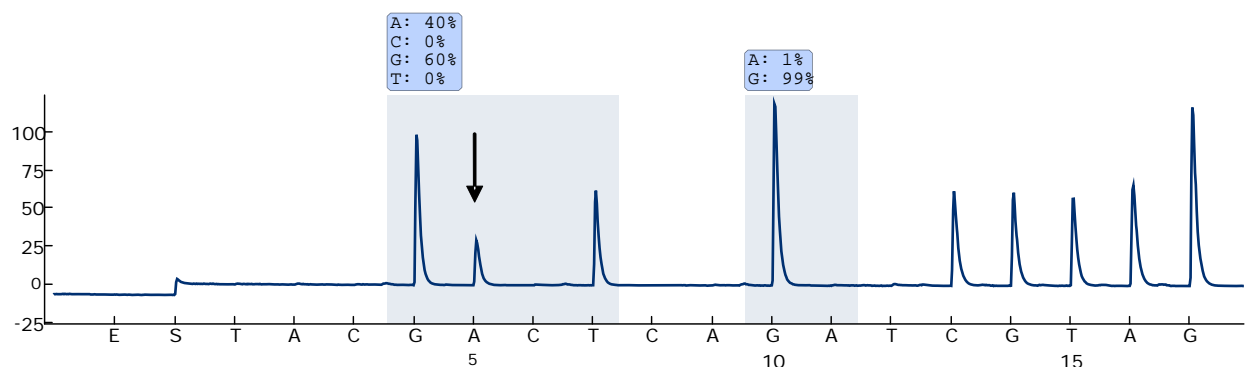
Representative Pyrogram results are shown in Figures 7–11.



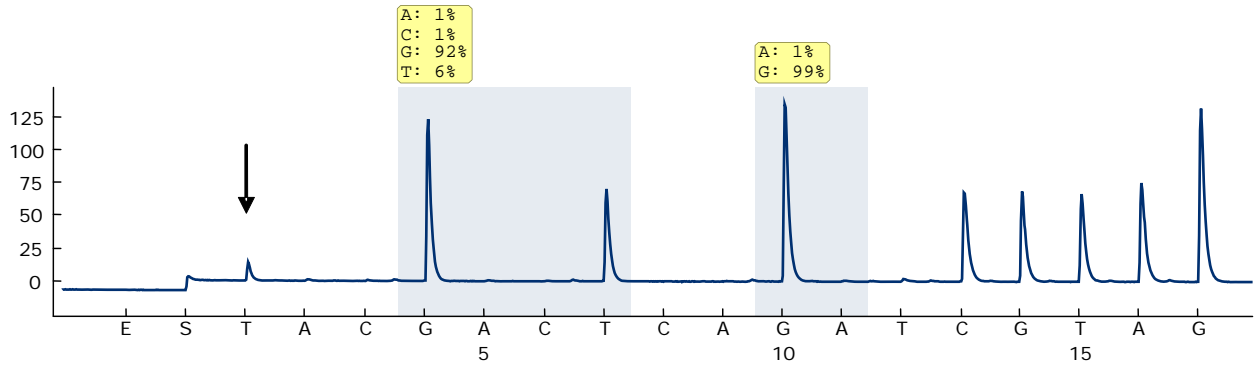
**Figure 7. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codons 12 and 13.**



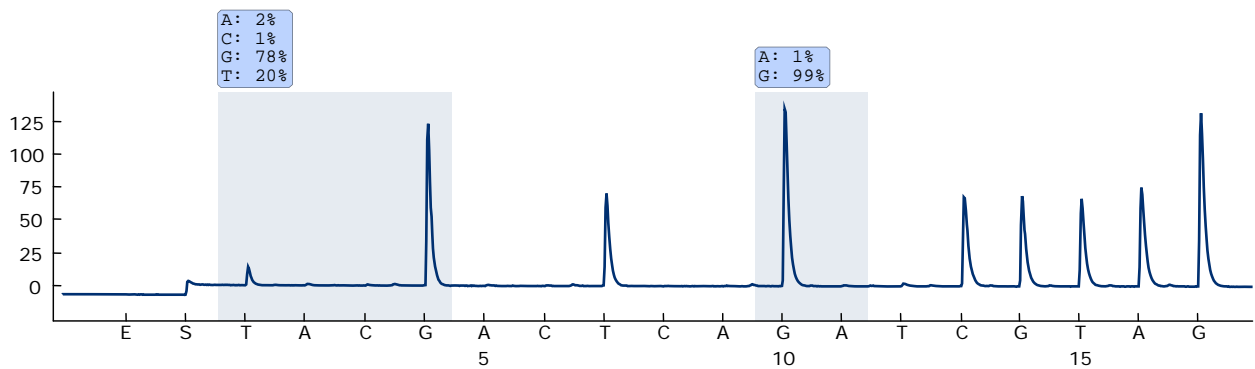
**Figure 8. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in codon 61.**



**Figure 9. Pyrogram trace obtained after analysis of samples with a GGT → GAT mutation in base 2 of codon 12 (nucleotide 35, indicated with an arrow).**



**Figure 10. Pyrogram trace obtained after analysis of samples with a GGT → TGT mutation in base 1 of codon 12 (nucleotide 34, indicated with an arrow) with the “Sequence to Analyze” GNTGRCGTAGGC targeting base 2 in codon 12 (nucleotide 35). A yellow color indicates that this sequence is unexpected and needs to be checked.**



**Figure 11. Pyrogram trace and result obtained after reanalysis of the sample in Figure 10. The mutation GGT → TGT was reanalyzed with the “Sequence to Analyze” NGTGRCGTAGGC targeting base 1 in codon 12 (nucleotide 34).**

## Troubleshooting guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: [www.qiagen.com/FAQ/FAQList.aspx](http://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](http://www.qiagen.com)).

**Note:** Refer to the *PyroMark Q24 User Manual* for general troubleshooting of the instrument.

### Comments and suggestions

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#### 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 <sup>®</sup> 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> |
|--------------------|--|

## Comments and suggestions

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- |  |  |
|--|--|
| b) Mutation not defined in "Sequence to Analyse"         | Adjust the sequence to analyze in the assay setup (see Appendix A, page 44) 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 plug-in report. These samples should be analyzed manually using the PyroMark Q24 Software and 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.             |

### High background

- |   |   |
|---|---|
| a) Incorrect storage of nucleotides                               | Store nucleotides at 2–8°C. Storage at –15 to –30°C can cause an increase in the background.                            |
| b) Short cooling time of samples prior to Pyrosequencing analysis | Keep the samples on a PyroMark Q24 Plate Holder at room temperature for 10–15 minutes. Do not shorten the cooling time. |
| c) Contamination of cartridge                                     | Carefully clean the cartridge as described in the product sheet. Store the cartridge protected from light and dust.     |

### No signals in positive control (unmethylated control DNA)

- |   |  |
|---|--|
| a) Insufficient enzyme or substrate mix for all wells | Make sure to fill the PyroMark Q24 Cartridge according to the "Pre Run Information" in the "Tools" menu. |
| b) Reagents incorrectly stored or diluted             | Prepare the PyroMark Q24 Gold reagents according to the instructions supplied with the reagents.         |

## Comments and suggestions

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- |   |   |
|---|---|
| c) Insufficient activation of HotStarTaq DNA polymerase | The HotStarTaq DNA polymerase in the PyroMark PCR Master Mix requires an activation step of 15 minutes at 95°C.   |
| d) 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 *therascreen* KRAS 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".  $\alpha$ - and  $\beta$ -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 mutation GGT → GTT in codon 12

For this mutation, blank measurements were consistently close to 0 % units (n=72) resulting in 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 this position was set to 1 % unit, which is clearly above the consistent baseline level (LOB) of 0 % unit. When analyzing a sample with a mutation level of 7%, 95% of results (n=89) gave a signal that can be regarded as positive ( $\geq$ LOD, i.e.,  $\geq$ 1 % unit).

**Table 9. LOB and LOD determined for specific mutations**

<b>Nucleic acid substitution</b>	<b>Amino acid substitution</b>	<b>LOB (% units)</b>	<b>LOD (% units)</b>	<b>COSMIC ID* (V42)</b>
<b>Codon 12 (GGT)</b>				
GAT	G12D	0.6	2.2	521
GTT	G12V	0.0	1.0 (7) <sup>†</sup>	520
TGT	G12C	0.5	2.1	516
AGT	G12S	0.4	1.9	517
GCT	G12A	0.7	2.3	522
CGT	G12R	0.3	1.8	518
<b>Codon 13 (GGC)</b>				
GAC	G13D	0.3	1.9	532
<b>Codon 61 (CAA), as assayed in reverse orientation (TTG)</b>				
GTG	Q61H	0.8	2.8	554
TAG	Q61L	1.2	3.1	553
TCG	Q61R	1.6	3.5	552
ATG	Q61H	0.7	2.6	555
TTC	Q61E	1.2	3.1	550

\* From the Catalogue of Somatic Mutations in Cancer, available online at the Sanger Institute at [www.sanger.ac.uk/genetics/CGP/cosmic/](http://www.sanger.ac.uk/genetics/CGP/cosmic/).

<sup>†</sup> 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 wild type or mutant sequence were used as template for PCR amplification.

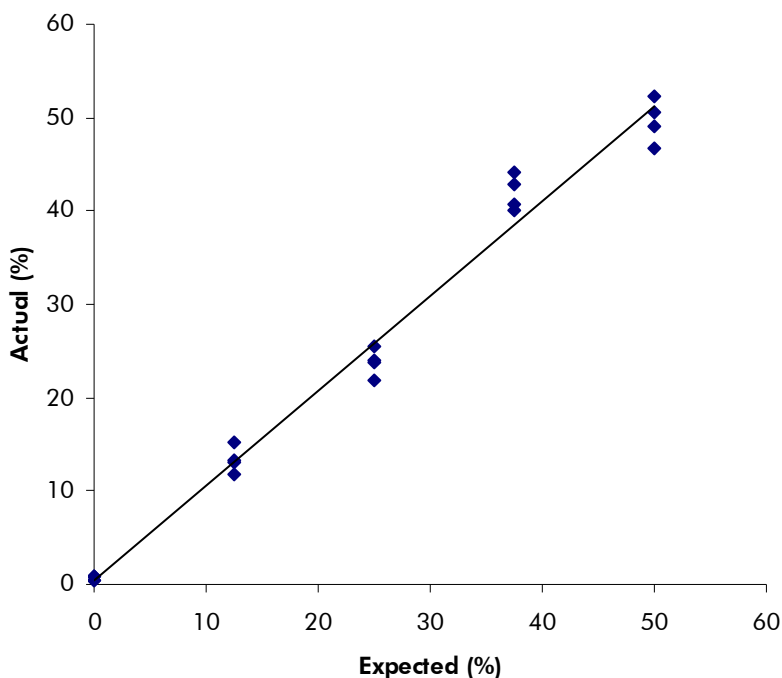
**Note:** The KRAS Plug-in Report algorithm was used to generate the LOB and LOD data. Manual analysis using the PyroMark Q24 Application Software as described in Protocol 6 (page 28) may result in slightly different values.

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

## Linearity

Linearity was measured according to CLSI Guideline EP6-A “Evaluation of the linearity of quantitative measurement procedures: a statistical approach; approved guideline”.

Plasmids carrying wild type and mutant sequences were mixed in proportions to give the following levels of mutation: 0, 12.5, 25, 37.5, and 50%. Four replicates of the mixes were placed in a random pattern on a plate and analyzed. The results for the mutation GGT → TGT in codon 12 were analyzed using Analyse-it® Software v2.04 (Analyse-it Software, Ltd., UK) and are shown in Figure 12.



**Figure 12. Linearity of mutation GGT → TGT in codon 12.**

The overall repeatability was 1.64 % units and the results were linear within an allowable nonlinearity of 3 % units. Similar results were obtained for the mutation GGC → GAC in codon 13.

## Intermediate precision

Determination of the linearity of the mutation GGT → TGT in codon 12 was repeated by 3 operators on 3 separate days using different combinations of PyroMark Q24 instruments and reagents. The results of the 3 runs are shown in Table 12.



**Table 12. Intermediate precision\***

% mutated plasmid <sup>†</sup>	Run 1		Run 2		Run 3		Summary	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.0	0.6	0.2	1.7	0.7	0.7	0.2	1.0	0.6
12.5	13.3	1.5	16.2	1.9	14.6	3.0	14.7	1.4
25.0	23.8	1.4	26.8	2.4	26.9	2.9	25.8	1.8
37.5	42.0	1.9	41.7	0.5	38.5	2.6	40.7	2.0
50.0	49.7	2.4	50.5	1.8	49.1	4.8	49.8	0.7

\* All values are given as % units. SD: standard deviation.

<sup>†</sup> Based on OD<sub>260</sub> measurement.

The values for the intermediate precision (SD) were therefore 0.6–2.0 % units in the measured range of 0–50% mutation level.

## Diagnostic evaluation

The *therascreen* KRAS Pyro Kit was evaluated in comparison with the DxS KRAS Mutation Kit. DNA was extracted from 100 formalin fixed paraffin embedded (FFPE) prospective colorectal cancer tumor samples and analyzed for mutations in codons 12 and 13.

DNA for testing was isolated using the EZ1 DNA Tissue Kit and analysis was carried out with the *therascreen* KRAS Pyro Kit on the PyroMark Q24 and with the DxS KRAS Mutation Kit on the ABI PRISM<sup>®</sup> 7900HT SDS.

Of the 100 samples analyzed, mutational status could be determined in 91 samples with the DxS KRAS Mutation Kit. With the *therascreen* KRAS Pyro Kit it was possible to determine the mutational status for 94 samples.

Excluding samples that failed with one or both kits, the *therascreen* KRAS Pyro Kit and DxS KRAS Mutation Kit showed 100% concordance of results.

The diagnostic sensitivity of the *therascreen* KRAS Pyro Kit was 100% and the diagnostic specificity was 100% (Table 13).

**Table 13. Results of the analyzed prospective colorectal cancer tumor samples for codons 12 and 13**

		DxS KRAS Mutation Kit			
		Mutant	Wild type	Unknown	Total
<i>therascreen</i> KRAS Pyro Kit	Mutant	33	0	1	<b>34</b>
	Wild type	0	57	3	<b>60</b>
	Unknown	0	1	5	<b>6</b>
	Total	<b>33</b>	<b>58</b>	<b>9</b>	<b>100</b>

### Analysis of codon 61

The same 100 samples were analyzed for mutations in codon 61 using the *therascreen* KRAS Pyro Kit. Only one sample gave a failed quality assessment for the codon 61 assay. This sample also failed in both the *therascreen* KRAS Pyro Kit and DxS assays for codon 12 and 13, indicating that the DNA was of too low quality. The higher success rate for the codon 61 assay indicates that it is less dependent on the DNA quality than both the *therascreen* KRAS Pyro Kit and DxS assays for codon 12 and 13. Since the DxS assay does not test for mutations in codon 61, no direct comparison of the assays is possible.

Mutations in codon 61 were detected in 4 of the 99 samples. Three contained frequent mutations (CAC, CAT, CTA) in codon 61 while the fourth sample contained mutations in both codon 60 (GGT→GGA) and codon 61 (CAA→AAA).

**Note:** In all runs used for determination of performance characteristics the signal was over 60 RLU, as routinely obtained from 10 ng of DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue.

## References

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## Symbols



Contains reagents sufficient for <N> tests

<N>



Use by



In vitro diagnostic medical device



Catalog number



Lot number



Material number



Components



Contains



Number



Global Trade Item Number



Temperature limitation



Manufacturer



Consult instructions for use

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## Appendix A: Setting Up *therascreen* KRAS Pyro Assays


In case the KRAS Plug-in Report has been installed, pre-defined Assay Setups for codons 12 and 13 and codon 61 are available in the shortcut browser of the PyroMark Q24 Software under the path "Example Files/PyroMark Setups/KRAS". The following steps do not need to be performed. The KRAS Plug-in Report can be obtained by e-mail from [pyro.plugin@qiagen.com](mailto:pyro.plugin@qiagen.com).

We strongly recommend the use of the KRAS Plug-in Report over manual analysis. After installation of the plug-in or each time a new software is installed or upgraded on the office computer the correct function of the plug-in should be verified as described in the Plug-In Quick Guide.

If the KRAS Plug-in Report has not been installed, the assay file must be set up manually before running the *therascreen* KRAS Pyro assay for the first time. Set up the assay for KRAS codons 12 and 13, and KRAS codon 61 by using the PyroMark Q24 Software, as described below.

### Procedure

#### KRAS codons 12 and 13

1. Click  in the toolbar and select "New AQ Assay".
2. Type the following sequence in "Sequence to Analyze".

**GNTGRCGTAGGC**

**Note:** The most frequent mutations in codon 12 will be detected in nucleotide 35 (second position) using this "sequence to Analyze". To analyze if mutations are present in nucleotide 34 (first position), change the "Sequence to Analyze" to the following sequence.

**NGTGRCGTAGGC**

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

3. Manually enter the following "Dispensation Order".

**TACGACTCAGATCGTAG**

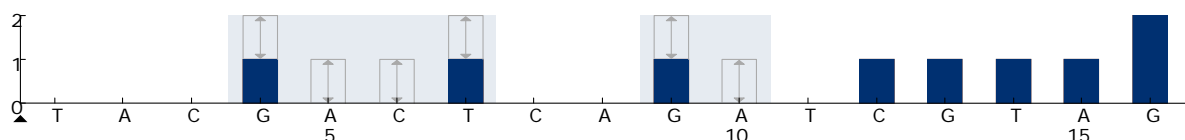


Figure 13. Histogram for codons 12 (nucleotide 35) and 13 (nucleotide 38) with the "Sequence to Analyze" **GNTGRCGTAGGC**.

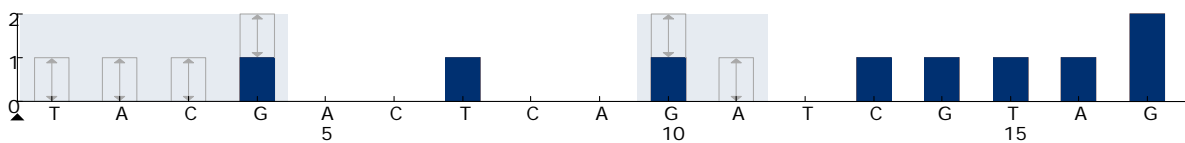



Figure 14. Histogram for codons 12 (nucleotide 34) and 13 (nucleotide 38) with the "Sequence to Analyze" NGTGRCGTAGGC.

4. Click the "Analysis Parameters" tab, and increase "Peak Height Threshold - Required peak height for Passed quality:" to 30.
5. Click  in the toolbar and save the assay as "KRAScodon 12+13".

### KRAS codon 61

6. Click  in the toolbar and select "New AQ Assay".
7. Type the following sequence in "Sequence to Analyze".  
**CTCDTGACCTG**

**Note:** The most frequent mutations in codon 61 will be detected in nucleotide 183 (third position) with this sequence to analyze. To analyze if mutations are present in nucleotide 182 (second position), change the "Sequence to Analyze" to the following sequence.

**CTCTHGACCTG**

To analyze if mutations are present in nucleotide 181 (first position), change the "Sequence to Analyze" to the following sequence.

**CTCTTSACCTG**

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

8. Manually add the following "Dispensation Order".  
**GCTCAGTCAGACT**

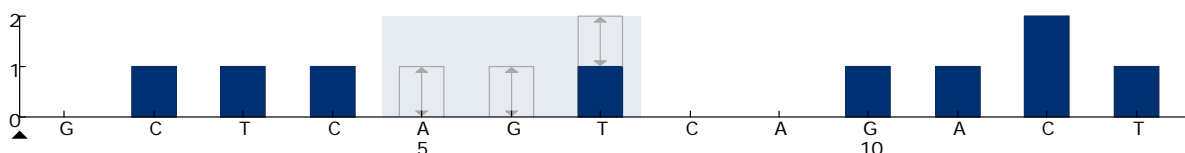


Figure 15. Histogram for codon 61 (nucleotide 183) with the "Sequence to Analyze" CTCDTGACCTG.

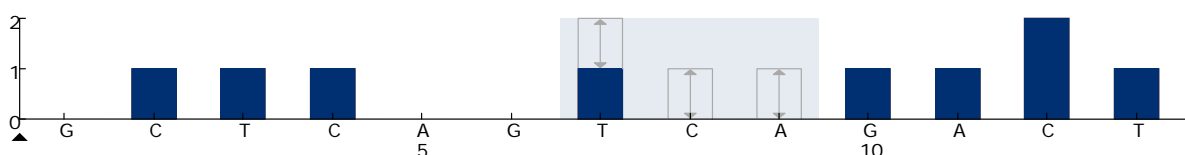
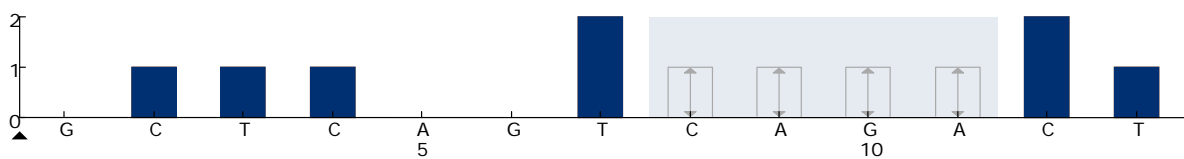



Figure 16. Histogram for codon 61 (nucleotide 182) with the "Sequence to Analyze" CTCTHGACCTG.



**Figure 17. Histogram for codon 61 (nucleotide 182) with the "Sequence to Analyze" CTCTTSACCTG.**

- 9. Click the "Analysis Parameters" tab and increase "Peak Height Threshold - Required peak height for Passed quality:" to 30.**
- 10. Click  in the toolbar and save the assay as "KRAScodon 61".**

## Appendix B: Emptying the Waste Container and Troughs

<p><b>WARNING</b></p> 	<p><b>Hazardous chemicals</b></p> <p>The Denaturation Solution used with the vacuum workstation contains sodium hydroxide, which is irritating to eyes and skin. Always wear safety glasses, gloves, and a lab coat.</p> <p>The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Safety Data Sheets (SDSs) or OSHA,<sup>*</sup> ACGIH,<sup>†</sup> or COSHH<sup>‡</sup> 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 (Milli-Q 18.2 MΩ x cm, [www.millipore.com](http://www.millipore.com), or equivalent).

### Procedure

1. **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.**
2. **Discard any solutions left in the troughs.**
3. **Rinse the troughs with high-purity water or replace them if necessary**
4. **Empty the waste container.**

**Note:** The cap can be removed without disconnecting the tubing.

5. **If the vacuum workstation must be cleaned (for example, due to dust or spillage), follow the instructions in the *PyroMark Q24 User Manual*.**

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Product	Contents	Cat. no.
<i>therascreen</i> KRAS 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 $\alpha$ S, dCTP, dGTP, dTTP, and H <sub>2</sub> O	971460
PyroMark Q24 MDx	Sequence based detection platform for Pyrosequencing of 24 samples in parallel	9001513
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PyroMark Q24 MDx Software	Application software	9019063
PyroMark Q24 Software	Analysis software	9019062
<b>Accessories</b>		
PyroMark Q24 Plate (100)	24-well sequencing reaction plate	979301

\* UK only.

† Rest of world.



<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
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