

therascreen[®] GIST RapidScreen Pyro[®] Kit Handbook



Version 1

IVD

For in vitro diagnostic use



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Intended Use

The *therascreen* GIST RapidScreen Pyro Kit is an in vitro nucleic acid sequence-based detection test based on Pyrosequencing® technology for the quantitative detection of mutations in exon 9 of the human *KIT* gene and in exon 18 of the human *PDGFRA* gene in genomic DNA derived from human tissue samples.

The *therascreen* GIST RapidScreen Pyro Kit is intended to provide clinicians with information to aid the management of patients diagnosed with gastrointestinal stromal tumor (GIST) more likely to benefit from drugs targeting signaling pathways, such as imatinib. 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.

This product is not intended for use with samples from lung tissue.

Summary and Explanation

The *therascreen* GIST RapidScreen Pyro Kit is used for quantitative measurements of mutations in *KIT* exon 9 and *PDGFRA* exon 18 (see Figure 1). Detection of mutations in *KIT* exon 9 allows the use of the appropriate dose of imatinib and detection of mutations in *PDGFRA* exon 18 helps to exclude less sensitive or resistant genotypes (1–3).

<i>KIT</i> exon 9	ATGCTCTGCTTCTGTACTGCCAGTGGATGTGCAGACACTAAACTCATCTGGGCCACCGTTTGG AAAGCTAGTGGTTCAGAGTTCTATAGATTCTAGTGCATTCAAGCACAATGGCACGGTTGAATG TAAGGCTTACAACGATGTGGGCAAGACTTCTGCCTATTTAACTTTGCATTTAAAGGTAACAA CAAAG
<i>PDGFRA</i> exon 18	TGTGTCCACCGTGATCTGGCTGCTCGCAACGTCTCCTGGCACAAGGAAAAATTGTGAAGATC TGTGACTTTGGCCTGGCCAGAGACATCATGCATGATTCTGAACTATGTGTGCGAAAGGCAGT

Figure 1. Genomic context of the sequenced regions of the human *KIT* and *PDGFRA* genes (Ensembl IDs ENSG00000157404 and ENSG00000134853). Codon 503 in the *KIT* gene and codon 842 in the *PDGFRA* gene are indicated by squares.

The kit consists of two assays: one for detecting mutations in *KIT* exon 9 and the other for detecting mutations in *PDGFRA* exon 18 (see Figure 2). 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.

Note: Both assays are sequenced in the forward direction.

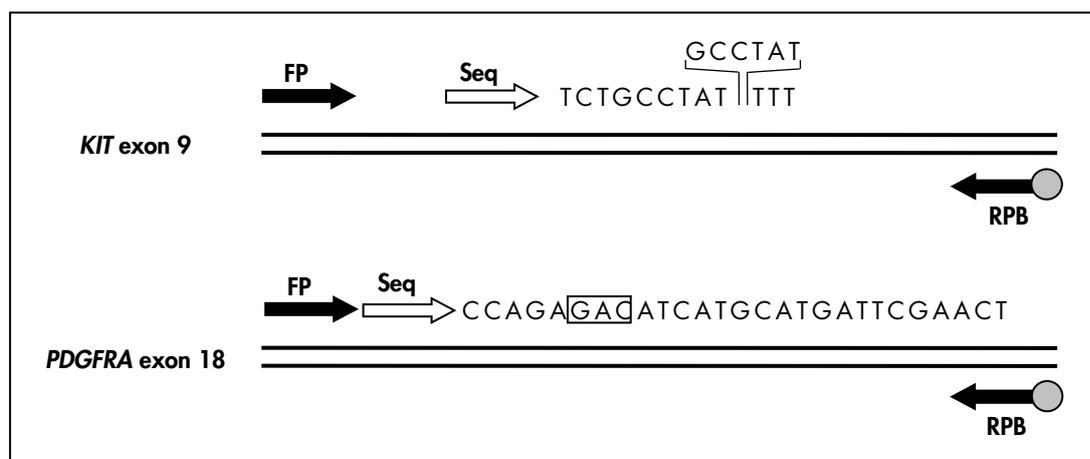


Figure 2. Illustration of the *KIT*/*PDGFRA* assays. The sequence indicated is the analyzed sequence for a wild-type sample. The position and sequence of the 6 bp duplication in *KIT* exon 9 is indicated. The square indicates codon 842 of *PDGFRA* exon 18. **FP:** Forward PCR primers; **RPB:** Reverse PCR primers (B indicates biotinylation); **Seq:** Sequencing primers.

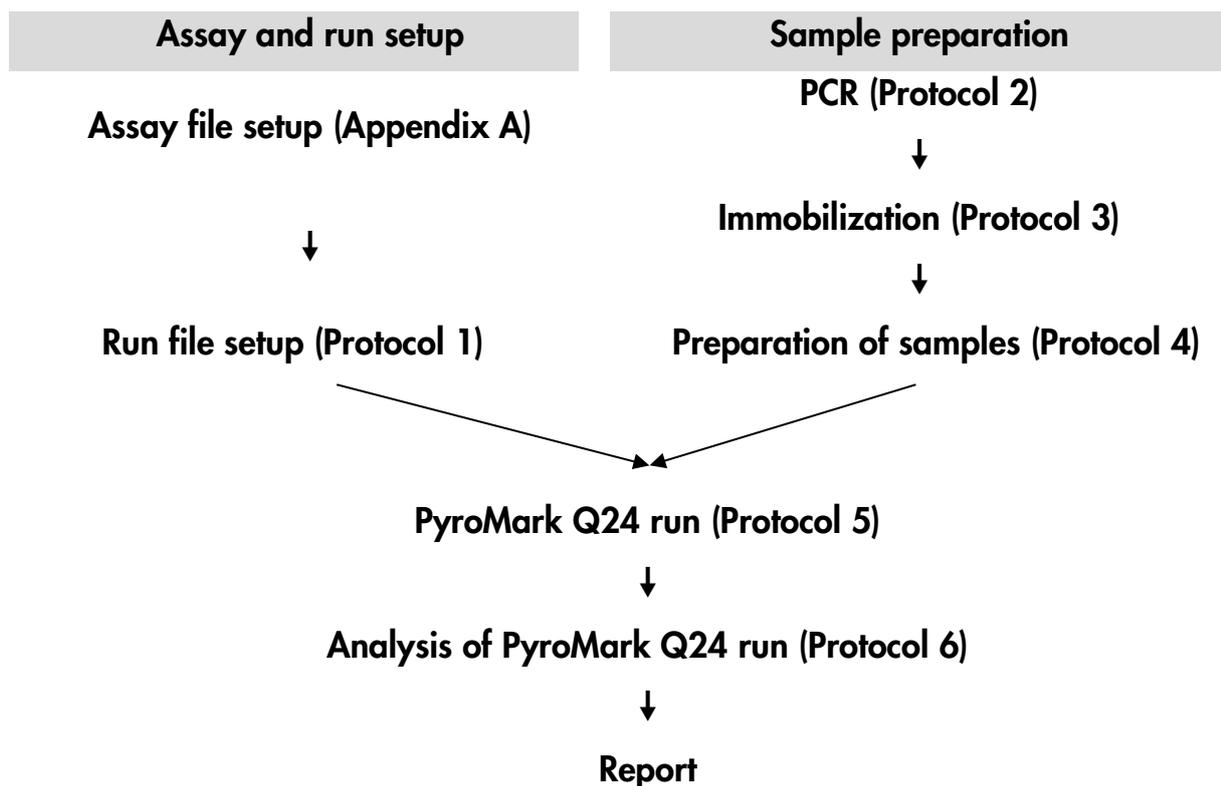
The product consists of a PCR primer mix and a sequencing primer for each assay. The primers are delivered in solution. Each vial contains 32 µl of each primer or primer mix.

Principle of the Procedure

The workflow below illustrates the assay procedure. After PCR using primers targeting *KIT* exon 9 and *PDGFRA* exon 18, the amplicons are immobilized on Streptavidin Sepharose® High Performance beads. Single-stranded DNA is prepared, and the corresponding sequencing primers anneal to the DNA. The samples are then analyzed on the PyroMark Q24 using assay setup files and a run file.

It is recommended to use the GIST RapidScreen Plug-in Report to analyze the run. The GIST RapidScreen Plug-in Report can be obtained by email from pyro.plugin@qiagen.com. However, the run can also be analyzed using the analysis tool integral to the PyroMark Q24 system. The "Sequence to Analyze" can be adjusted for detection of rare mutations after the run (see "Protocol 6: Analysis of a PyroMark Q24 run", page 29, and "Appendix A: Setting Up *therascreen* GIST RapidScreen Pyro Assays", page 48).

Workflow of *therascreen* GIST RapidScreen 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 33). 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 GIST RapidScreen Pyro Kit (box 1/2)

<i>therascreen</i> GIST RapidScreen Pyro Kit (24)	(24)
Catalog no.	971510
Number of reactions	24
Seq Primer KIT exon 9	32 µl
Seq Primer PDGFRA exon 18	32 µl
PCR Primer Mix KIT exon 9	32 µl
PCR Primer Mix PDGFRA exon 18	32 µl
PyroMark PCR Master Mix, 2x	850 µl
CoralLoad® Concentrate, 10x	1.2 ml
H ₂ O	3 x 1.9 ml
Unmethylated Control DNA, 10 ng/µl	100 µl

therascreen Pyro Buffers and Reagents (box 2/2)

therascreen Pyro 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	1 180 μ l
dCTP	1 180 μ l
dGTP	1 180 μ l
dTTP	1 180 μ l
therascreen <i>GIST RapidScreen Pyro Kit Handbook</i> (English)	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.

Reagents

- DNA isolation kit (see "DNA isolation", page 15)
- Streptavidin Sepharose High Performance (GE Healthcare, cat. no. 17-5113-01; www.gelifesciences.com)
- High-purity water (Milli-Q[®] 18.2 M Ω 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%) *

Consumables

- Sterile pipet tips (with filters for PCR setup)
- 24-well PCR plates (see “Recommended 24-well plates”, page 12)
- Adhesive foil

Equipment

- Pipets (adjustable)[†]
- Benchtop microcentrifuge[†]
- Thermal cycler[†] and appropriate PCR tubes
- PyroMark Q24 (cat. no. 9001513 or 9001514)^{†‡}
- PyroMark Q24 Software (cat. no. 9019063 or 9019062)[‡]
- PyroMark Q24 Plate (cat. no. 979201)[‡]
- PyroMark Q24 Cartridge (cat. no. 979202)[‡]
- PyroMark Q24 Vacuum Workstation (cat. no. 9001515 or 9001517)^{†‡}
- Plate mixer[†] for immobilization to beads (see “Recommended plate mixers”, page 12)
- Heating block[†] capable of attaining 80°C

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

[†] 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.

Recommended 24-well plates

The 24-well plates in Table 1 are recommended for use with the *therascreen* GIST RapidScreen Pyro Kit.

Table 1. 24-well plates recommended for use with the *therascreen* GIST RapidScreen Pyro Kit

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

Recommended plate mixers

The orbital plate mixers in Table 2 are recommended for use with the *therascreen* GIST RapidScreen Pyro Kit.

Table 2. Plate mixers recommended for use with the *therascreen* GIST RapidScreen 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 safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN® kit and kit component.

The following hazard and precautionary statements apply to components of the *therascreen* GIST RapidScreen 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.

General precautions

The user should always pay attention to the following:

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

Reagent Storage and Handling

The *therascreen* GIST RapidScreen Pyro Kit is shipped in two boxes. The *therascreen* GIST RapidScreen Pyro Kit (box 1/2) is shipped on dry ice. PyroMark PCR Master Mix, CoralLoad Concentrate, unmethylated control DNA, and all primers should be stored at -15 to -30°C upon arrival.

The *therascreen* Pyro Buffers and Reagents (box 2/2) containing Buffers, Enzyme Mixture, Substrate Mixture, dATP α S, dCTP, dGTP, and dTTP (the reagents for Pyrosequencing analysis) is shipped on cool packs. These components should be stored at 2 – 8°C upon arrival. To minimize loss of activity, it is advisable to keep both the enzyme mixture and the substrate mixture in the vials supplied.

Reconstituted enzyme and substrate mixtures are stable for at least 10 days at 2 – 8°C . Reconstituted enzyme and substrate mixtures can be frozen and stored in their vials at -15 to -30°C . Frozen reagents should not be subjected to more than 6 freeze–thaw cycles.

Note: Nucleotides should not be frozen.

The *therascreen* GIST RapidScreen Pyro Kit is stable until the kit expiration date when stored under these conditions.

Specimen Handling and Storage

All samples must be treated as potentially infectious material.

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

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

Procedure

DNA isolation

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

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

Table 3. DNA purification kits recommended for use with the *therascreen* GIST RapidScreen Pyro Kit

Sample material	Nucleic acid isolation kit	Catalog number (QIAGEN)
Paraffin-embedded tissue	QIAamp DNA FFPE Tissue Kit (50)	56404
	EZ1 DNA Tissue Kit (48)*	953034
Blood	QIAamp DSP DNA Blood Mini Kit [†]	61104

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

[†] 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 GIST RapidScreen Plug-in Report has not been installed, create an Assay Setup (see “Appendix A: Setting Up *therascreen* GIST RapidScreen Pyro Assays”, page 48). This must only be done once before running the *therascreen* GIST RapidScreen Pyro assays for the first time. In case the GIST RapidScreen Plug-in Report has been installed, predefined Assay Setups are available in the shortcut browser of the PyroMark Q24 software under the path “Example Files/PyroMark Setups/GIST”. The GIST RapidScreen Plug-in Report can be obtained by email from pyro.plugin@qiagen.com.

Procedure

1. **Click  in the toolbar.**
A new run file is created.
2. **Enter the run parameters (see “Run parameters”, page 17).**
3. **Set up the plate by adding assays for both KIT exon 9 and PDGFRA exon 18 to wells corresponding to the samples to analyze.**
Note: A negative control sample (without template DNA) should be included in every PCR setup for at least one assay.
Note: Include a sample with unmethylated control DNA for each assay in every Pyrosequencing run (“Controls”, page 7).
4. **When the run is set up and ready to run on the PyroMark Q24 System, print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Select “Pre Run Information” from the “Tools” menu and, when the report appears, click .**
5. **Close the run file and copy it to a USB stick (supplied with the system) using Windows® Explorer.**
The printed Pre Run Information can be used as a template for the sample setup (see “Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads”, page 21).

To run the plate on PyroMark Q24, see “Protocol 5: Running the PyroMark Q24”, page 27.

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> GIST RapidScreen Pyro Kit box 1 and box 2 to be used. The lot number can be found on the product label. Note: We recommend entering both lot numbers so that any unexpected problems with the <i>therascreen</i> GIST RapidScreen Pyro Kit can be traced.
Run note:	Optional: Enter a note about the contents or purpose of the run.

Add assay files

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

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

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

Enter sample IDs and notes

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

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

Protocol 2: PCR using the PCR reagents supplied with the *therascreen* GIST RapidScreen Pyro Kit

This protocol is for PCR amplifications of a region containing *KIT* exon 9 and a separate PCR amplification of a region containing *PDGFRA* exon 18 using the *therascreen* GIST RapidScreen Pyro Kit.

Important points before starting

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

Things to do before starting

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

Procedure

1. Thaw all necessary components (see Table 4).

Mix well before use.

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

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

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

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

Component	Volume/reaction
PyroMark PCR Master Mix, 2x	12.5 μ l
CoralLoad Concentrate, 10x	2.5 μ l
PCR Primer KIT exon 9 or PCR Primer PDGFRA exon 18	1 μ l
Water (H ₂ O, supplied)	4 μ l
Total volume	20 μl

3. Mix the reaction mix thoroughly, and dispense 20 μ l into each PCR tube.

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

4. Add 5 μ l template DNA (2–10 ng of genomic DNA) to the individual PCR tubes (Table 5), 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 5. Preparation of PCR

Component	Volume/reaction
Reaction mix	20 μ l
Sample DNA	5 μ l
Total volume	25 μl

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

Table 6. Optimized cycling protocol

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

- 6. Place the PCR tubes in the thermal cycler and start the cycling program.**
- 7. After amplification, proceed with "Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads", page 21.**
The PCR samples can be stored at 2–8°C for up to 3 days.

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

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

Things to do before starting

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

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

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

Procedure

- 1. Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.**
- 2. Prepare a master mix for DNA immobilization according to Table 7.**
Prepare a volume greater than that required for the total number of reactions to be performed (for the number of reactions + one extra).

Table 7. Master mix for DNA immobilization

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

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

- 3. Add 70 μ l of the master mix to wells of a 24-well PCR plate as predefined in the run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 16).**

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

- 4. Add 10 μ l of biotinylated PCR product from Protocol 2 to each well containing master mix as predefined in the run setup (see “Protocol 2: PCR using the PCR reagents supplied with the *therascreen* GIST RapidScreen Pyro Kit”, page 18).**

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

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

Ensure that no leakage is possible between the wells.

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

During this step, proceed immediately with “Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24”, page 23.

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 16), depending on the region of analysis (*KIT* exon 9 or *PDGFRA* exon 18).
- Do not shorten the time for cooling down the samples after heating to 80°C.
- Perform the function test for the filter probes as described in the *PyroMark Q24 User Manual* on a regular basis and exchange filter probes when indicated.

Procedure

- 1. Dilute a sufficient amount of each sequencing primer, Seq Primer *KIT* exon 9 and Seq Primer *PDGFRA* exon 18, in PyroMark Annealing Buffer as shown in Table 8.**

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

Do not dilute and store more sequencing primer.

Table 8. Example dilution of the sequencing primers

Component	Volume/sample	Volume for 9 + 1 reactions
PyroMark Annealing Buffer	24.2 μ l	242 μ l
Seq Primer KIT exon 9 or Seq Primer PDGFRA exon 18	0.8 μ l	8 μ l
Total volume	25 μl	250 μl

- 2. Add 25 μ l of diluted sequencing primer to each well of the PyroMark Q24 Plate according to the run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 16).**

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

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

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



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

5. **Apply vacuum to the tool by opening the vacuum switch.**
6. **Slowly lower the filter probes of the vacuum tool into the PCR plate to capture the beads containing immobilized template. Hold the probes in place for 15 seconds. Take care when picking up the vacuum tool.**
 Sepharose beads sediment quickly. Capturing of beads must take place immediately following agitation. If more than 1 minute has elapsed since the plate was agitated, agitate again for 1 minute before capturing the beads.
 Inspect the PCR plate for complete take up of all samples by the vacuum tool.
7. **Transfer the vacuum tool to the trough containing 40 ml 70% ethanol (trough 1; Figure 3). Flush the filter probes for 5 seconds.**
8. **Transfer the vacuum tool to the trough containing 40 ml Denaturation Solution (trough 2; Figure 3). Flush the filter probes for 5 seconds.**
9. **Transfer the vacuum tool to the trough containing 50 ml Wash Buffer (trough 3; Figure 3). Flush the filter probes for 10 seconds.**
10. **Raise the vacuum tool up and back, beyond 90° vertical, for 5 seconds to drain liquid from the filter probes (Figure 4).**



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

11. **While the vacuum tool is held over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).**
12. **Release the beads into the PyroMark Q24 Plate by lowering the filter probes into the diluted sequencing primer and moving the vacuum tool gently from side to side.**
 Take care not to damage the surface of the PyroMark Q24 Plate by scratching it with the filter probes.
13. **Transfer the vacuum tool to the trough containing high-purity water (trough 4; Figure 3) and agitate it for 10 seconds.**
14. **Wash the filter probes by lowering the probes into high-purity water (trough 5; Figure 3) and applying vacuum. Flush the probes with 70 ml high-purity water.**

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

At the end of a 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: Emptying the Waste Container and Troughs”, page 51.
- 18. Heat the PyroMark Q24 Plate with the samples at 80°C for 2 minutes using the prewarmed PyroMark Q24 Plate Holder.**
- 19. Remove the PyroMark Q24 Plate from the hot plate holder, and place it on a second PyroMark Q24 Plate Holder that was kept at room temperature (15–25°C) to let the samples cool to room temperature for 10–15 minutes.**
- 20. Proceed with “Protocol 5: Running the PyroMark Q24”, page 27.**

Protocol 5: Running the PyroMark Q24

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

Important points before starting

- The Pre Run information report, found in the “Tools” menu at run setup (see “Protocol 1: Run setup for the PyroMark Q24 system”, page 16), provides information about the volume of nucleotides, enzyme, and substrate buffer needed for a specific run.
- Use disposable tips without hydrophobic filters for loading the cartridge to permit correct function of the cartridge.

Procedure

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

Do not vortex!

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

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

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

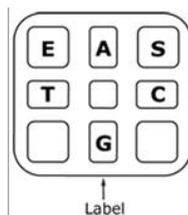


Figure 5. Illustration of the PyroMark Q24 Cartridge as seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (E), substrate mixture (S), and nucleotides (A, T, C, G) according to the volume information given in the Pre Run information report, found in the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

6. **Open the cartridge gate and insert the filled reagent cartridge with the label facing out. Push the cartridge in fully and then push it down.**
7. **Ensure the line is visible in front of the cartridge and close the gate.**
8. **Open the plate-holding frame and place the plate on the heating block.**
9. **Close the plate-holding frame and the instrument lid.**
10. **Insert the USB stick (containing the run file) into the USB port at the front of the instrument.**
Do not remove the USB stick before the run is finished.
11. **Select “Run” in the main menu (using the ▲ and ▼ screen buttons) and press “OK”.**
12. **Select the run file using the ▲ and ▼ screen buttons.**
To view the contents of a folder, select the folder and press “Select”. To go back to the previous view, press “Back”.
13. **When the run file is selected, press “Select” to start the run.**
14. **When the run is finished and the instrument confirms that the run file has been saved to the USB stick, press “Close”.**
15. **Remove the USB stick.**
16. **Open the instrument lid.**
17. **Open the cartridge gate and remove the reagent cartridge by lifting it up and pulling it out.**
18. **Close the gate.**
19. **Open the plate-holding frame and remove the plate from the heating block.**
20. **Close the plate-holding frame and the instrument lid.**
21. **Discard the plate and clean the cartridge, as per the instructions in the product sheet supplied with the cartridge.**
22. **Analyze the run according to “Protocol 6: Analysis of a PyroMark Q24 run”, page 29.**

Protocol 6: Analysis of a PyroMark Q24 run

This protocol describes the mutation analysis of a finished GIST RapidScreen 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 GIST RapidScreen Plug-in Report, go to step 5. If using the AQ analysis integral to the PyroMark Q24 Software, go to step 6.

Note: We strongly recommend using the GIST RapidScreen Plug-in Report for documentation and interpretation of results. The GIST RapidScreen Plug-in Report can be obtained by email from pyro.plugin@qiagen.com. This report ensures that the respective LOD values (Table 9) and different "Sequence to Analyze" are used to automatically detect all mutations.

Note: Two complex mutations in *PDGFRA* exon 18 (2526_2538>G and 2524_2526 GAC>TAT) cannot be analyzed using the AQ analysis in the PyroMark Q24 Software. We recommend to use the GIST RapidScreen Plug-in Report for analysis of *PDGFRA* exon 18 complex mutations

5. **Using the GIST RapidScreen Plug-in Report:**
To generate a report, select "AQ Add On Reports/GIST" from "Reports" in the menu (see Figure 6).

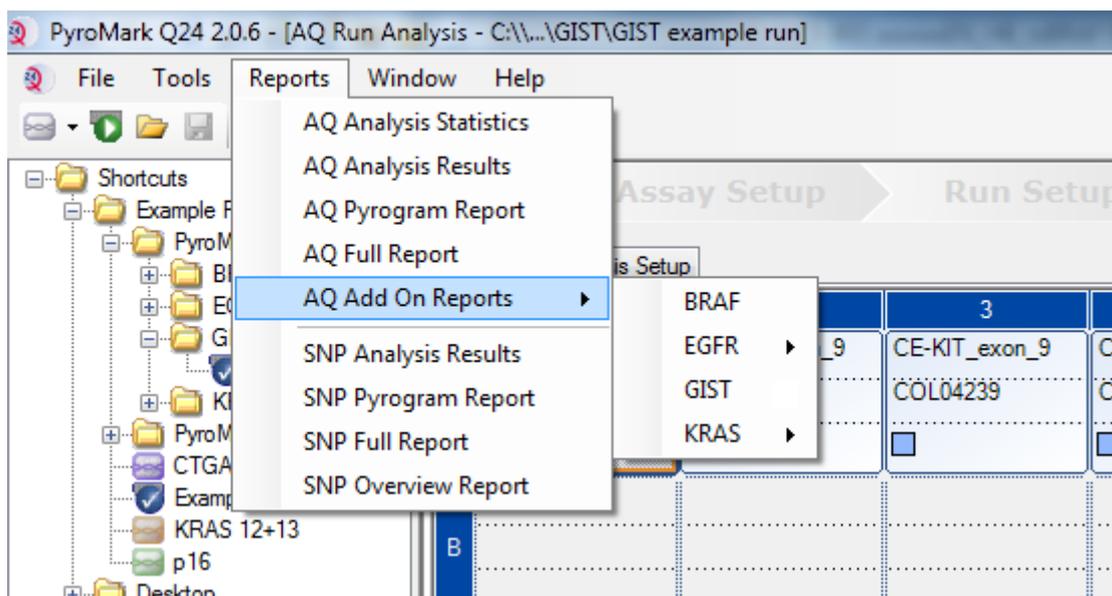


Figure 6. GIST RapidScreen Plug-in Report menu.

The wells will automatically be analyzed for all mutations for which LOD is given in Table 9. The results will be presented in an overview table (see Figure 7), followed by the detailed results which include Pyrograms and analysis quality.

Summary

Well	Assay Name	Sample ID	Result	Frequency [% units]	Nucleotide Substitution	Amino Acid Substitution	Info
A1	cKIT Exon 9	COL04237	No mutation detected				
A2	cKIT Exon 9	COL04238	Mutation	51,6	1509_1510insGCCTAT	Y503_F504insAY	
A3	cKIT Exon 9	COL04239	Mutation	29,6	1509_1510insGCCTAT	Y503_F504insAY	
A4	cKIT Exon 9	COL04240	No mutation detected				
A5	cKIT Exon 9	wt control DNA	No mutation detected				
A8	cKIT Exon 9		Failed Analysis				⚠
C1	PDGFRA Exon 18	COL04237	No mutation detected				
C2	PDGFRA Exon 18	COL04238	Potential low level mutation	4,5	2525A>T	D842V	⚠
C3	PDGFRA Exon 18	COL04239	No mutation detected				
C4	PDGFRA Exon 18	COL04240	Mutation	52,2	2524_2535del12 or 2526_2537del12	D842_H845del or I843_D846del	
C5	PDGFRA Exon 18	wt control DNA	No mutation detected				
C8	PDGFRA Exon 18		Failed Analysis				⚠

⚠ See detailed results below.

NOTE: The result must be validated by comparing the observed peaks with the expected peak heights displayed as grey bars. For further information about data evaluation and result interpretation please refer to the handbook.

Figure 7. GIST RapidScreen Plug-in Report.

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

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

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

Re-analysis of samples with no mutation detected with the standard “Sequence to analyze” or with “Check” or “Failed” quality assessment

The standard “Sequence to Analyze” as defined in the Analysis Setup addresses the 6 bp duplication in *KIT* exon 9 and the most frequent point mutation in codon 842 (GAC>GTC) of *PDGFRA* exon 18 (see Appendix A, page 48). If a sample contains a less frequent mutation in *PDGFRA* exon 18, the “Sequence to Analyze” can be changed to analyze the mutational status of this mutation, as described in Appendix A.

Two complex mutations in *PDGFRA* exon 18 (2526_2538>G and 2524_2526 GAC>TAT) cannot be analyzed using the AQ analysis in the PyroMark Q24 Software. We recommend to use the GIST RapidScreen Plug-in Report for analysis of *PDGFRA* exon 18 complex mutations

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 less frequent mutations go to “Analysis Setup” and change “Sequence to Analyze” to variants described in Appendix A or variants for other rare or unexpected mutations. Click “Apply”, and then click “To All” when the “Apply Analysis Setup” window appears.

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

Note: After changing “Sequence to Analyze”, ensure that the threshold for single peak height is set to 30 RLU.

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

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

Interpretation of Results

Interpretation of analysis results and detection of low-level mutations

It is strongly recommended that unmethylated control DNA is included in every run for comparison and as a control for background levels. The measured frequency of the control sample should be smaller or equal to the limit of blank (LOB). The LOB (limit of blank) and LOD (limit of detection) values given in the handbooks can be used when determining the presence of a mutation. These values were obtained using plasmid mixtures carrying the wild-type or the relevant mutated sequence.

After analysis using PyroMark Q24 Software or the Plug-in Reports, 3 results are possible.

- Mutation frequency $<$ LOD: Mutation not detected
- Mutation frequency $>$ LOD + 3 % units: Mutation
- Mutation frequency \geq LOD and \leq LOD + 3 % units: Potential low-level mutation

Note: If using the GIST RapidScreen Plug-in Report (see step 5 of “Protocol 6: Analysis of a PyroMark Q24 run”, page 29) and this occurs, a warning will be issued.

The range from LOD to LOD + 3 % units allows for sensitive detection of low-level mutations under optimal conditions. A measured frequency above LOB in the unmethylated control sample indicates a higher than usual level of background in the respective run, which may impact allele quantification, especially for low mutational levels. Therefore results with the “Potential low level mutation” warning must be carefully evaluated.

Samples with a reported potential low-level mutation should only be considered positive for the mutation if confirmed by rerunning in duplicate together with the unmethylated control DNA. The result of both duplicates should report the same mutation with values \geq LOD, and the control sample should report as “No mutation detected”. Otherwise the sample should be judged as “No mutation detected”.

Increased background for a mutation can be detected by comparing the LOB values listed in the handbook to the measurements obtained with the unmethylated control DNA. Samples with a reported potential low-level mutation can be rated as “Mutation not detected” without repetition if the measured frequency for the unmethylated control DNA is higher than the LOB value listed

in the handbook for the relevant mutation. Therefore 3 different scenarios are possible with reported potential low level mutations.

1. Measurement frequency with unmethylated control DNA >LOB for this mutation: Sample can be rated as "Mutation not detected" without repetition.
2. Result not reproduced in duplicate with the same result: Rate sample as "Mutation not detected".
3. Reproduced in duplicate with the same result and wild-type sample <LOB for relevant mutation: Mutation detected.

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. The Pyrograms should be examined for the appearance of unexpected peaks. 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. The failed result is not a basis for judgment of mutational status. For a valid mutation, a change in the peak height is always related to a corresponding change in height of another peak. A change in height of a single peak should not be rated as indicative for a mutation.

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

Note: A treatment decision for cancer patients must not be based solely on mutation status in *KIT* exon 9 and *PDGFRA* exon 18.

Table 9. LOB and LOD determined for specific mutations

Nucleic acid substitution	Amino acid substitution	LoB (% units)	LoD (% units)	COSMIC ID* (v58)
<i>KIT</i> exon 9				
1509_1510insGCCTAT	Y503_F504insAY	1.9	4.9	1326
<i>PDGFRA</i> exon 18				
2525A>T	D842V	0.6	3.6	736
2524G>T	D842Y [†]	0.6	3.6	12396
2524_2535 del12 or [‡]	D842_H845del or [‡]	2.2	5.2	737 or [‡]
2526_2537 del12	I843_D846del [‡]			96892
2527_2538 del12	I843_D846del [†]	3.0	6.0	12400
2528_2539 del12	I843_S847>T	4.2	7.2	12407
2530_2541 del12	M844_S847del	3.2	6.2	12402
2524_2532 del9	D842_M844del	1.5	4.5	12401
2524_2526 delGAC	D842del	0.9	3.9	12406
2526_2538 >G [§]	D842_D846>E	0.3	3.3	12408
2524_2526 GAC>TAT	D842Y [†]	0.9	3.9	12397

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

[†] The mutations 2524G>T and 2524_2526 GAC>TAT, and 2526_2537 del12 and 2527_2538 del12 result in the same amino acid change, respectively.

[‡] The mutations 2524_2535del12 and 2526_2537del12 result in the same nucleic acid change.

[§] The mutation 2526_2538 >G and 2524_2526GAC>TAT cannot be analyzed in the AQ mode of the PyroMark Q24 Software.

Representative results

Representative Pyrogram results are shown in Figures 8–11.

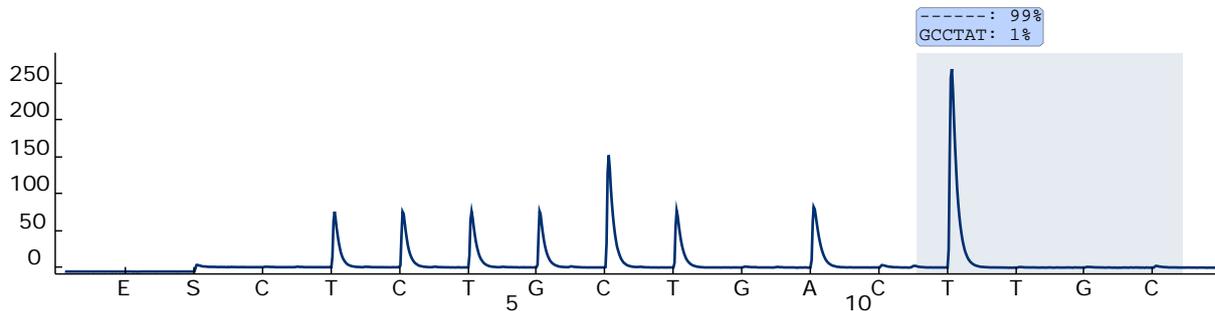


Figure 8. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in *KIT* exon 9 with the “Sequence to Analyze” TCTGCCTAT[GCCTAT]TTTA addressing the 6 bp duplication after codon 503.

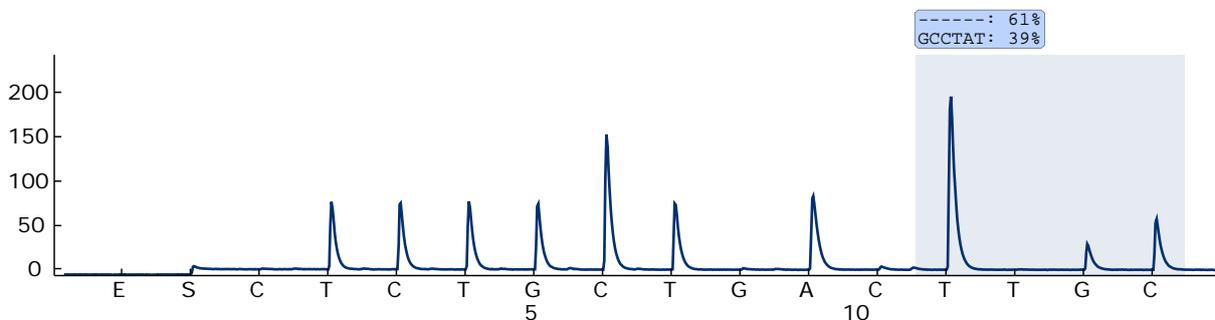


Figure 9. Pyrogram trace obtained after analysis of a sample with a GCCTAT duplication after codon 503 in *KIT* exon 9 with the “Sequence to Analyze” TCTGCCTAT[GCCTAT]TTA.

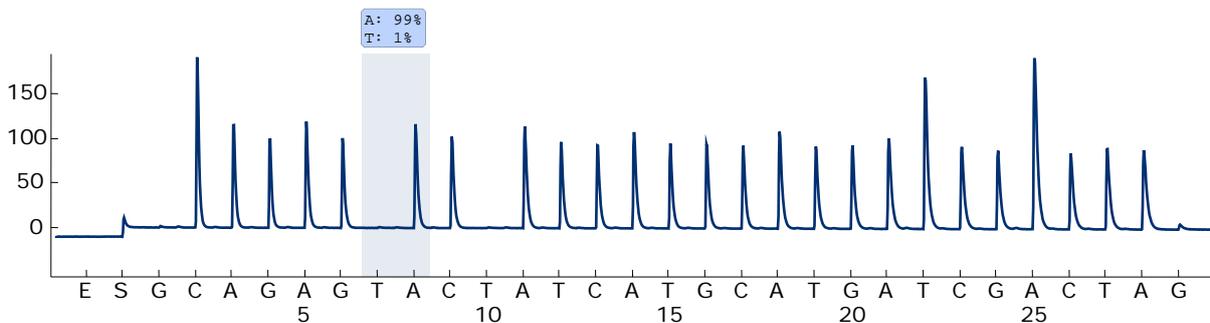


Figure 10. Pyrogram trace obtained after analysis of a sample with a wild-type genotype in *PDGFRA* exon 18 with the “Sequence to Analyze” CCAGAGWCATCATGCATGATTCTGA ACTAT addressing the mutation GAC>GTC in codon 842 (nucleotide 2525).

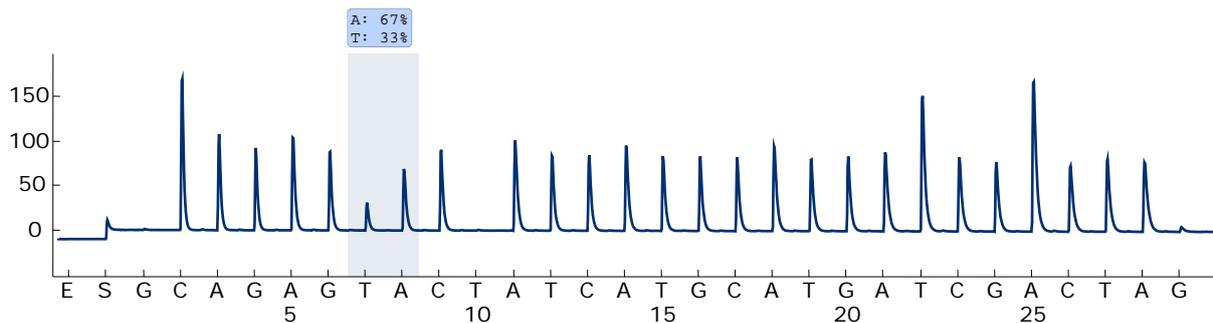


Figure 11. Pyrogram trace obtained after analysis of a sample with a GAC>GTC mutation in codon 842 (nucleotide 2525) in *PDGFRA* exon 18 with the “Sequence to Analyze” CCAGAGWCATCATGCATGATTCGAACTAT.

Troubleshooting guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

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

Comments and suggestions

Signals in the no template control (negative control)

- | | |
|-----------------------------|---|
| a) Cross-talk between wells | Signal from one well is detected in a neighboring well. Avoid placing samples with high signal intensities next to “no template control” wells. |
| b) PCR contamination | Use sterile pipet tips with filters. Store and extract materials such as specimens, controls, and amplicons separately from PCR reagents. |

Poor or unexpected sequence

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

Comments and suggestions

“Check” or “failed” result

- a) Low peak height
- Handling errors in PCR setup or sample preparation prior to Pyrosequencing can result in low peaks.
- It is important that the samples are completely taken up by the vacuum tool. Take care that the vacuum tool is lowered slowly into the samples and that the geometry of the PCR plate or strips used for immobilization allows complete take up of the samples.
- Perform the function test for filter probes as described in the *PyroMark Q24 Manual* on a regular basis and exchange filter probes when indicated.
- In case of a “Check” warning, carefully compare the Pyrogram to the histogram, which can be displayed by 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.
- b) Mutation not defined in the “Sequence to Analyze”
- Adjust the “Sequence to Analyze” in the assay setup (see “Appendix A: Setting Up *therascreen* GIST RapidScreen Pyro Assays”, page 48), and reanalyze the run.
- c) Unexpected rare mutation
- A “Check” or “Failed” quality assessment can be caused by an unexpected pattern of peaks. This might indicate an unexpected mutation, which is not analyzed by the provided “Sequence to Analyze”. These samples should be analyzed using the alternative “Sequences to Analyze” considering unexpected mutations.

Comments and suggestions

- 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 reagents according to the instructions in “Protocol 5: Running the PyroMark Q24”, page 27.
- c) PCR or sample preparation failure
- Handling errors in PCR setup, programming of the PCR cycler, or sample preparation prior to Pyrosequencing can result in no signals. Perform the function test for filter probes as described in the *PyroMark Q24 Manual* and exchange filter probes when indicated. Repeat the PCR and Pyrosequencing analysis.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the *therascreen* GIST RapidScreen 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.

Strict compliance with the user manual is required for optimal PCR results. Attention should be paid to expiration dates printed on the box and labels of all components. Do not use expired components.

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 10). LOB and LOD were determined according to the recommendations in the Clinical and Laboratory Standards Institute (CLSI) Guideline EP17-A "Protocol for determination of limits of detection and limits of quantitation; approved guideline". α - and β -errors (false positive and false negative, respectively) were set to 5%. LOD for some rare deletions in *PDGFRA* exon 18 were determined by adding 3 standard deviations of blank measurements to the LOB value. The LOD values were set at least 3 % units above the LOB value.

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

Table 10. LOB and LOD determined for specific mutations

Nucleic acid substitution	Amino acid substitution	LOB (% units)	LOD (% units)	COSMIC ID* (v58)
<i>KIT</i> exon 9				
1509_1510insGCCTAT	Y503_F504insAY	1.9	4.9	1326
<i>PDGFRA</i> exon 18				
2525A>T	D842V	0.6	3.6	736
2524G>T	D842Y†	0.6	3.6	12396
2524_2535 del12 or‡	D842_H845del or‡	2.2	5.2	737 or‡
2526_2537 del12	I843_D846del†			96892
2527_2538 del12	I843_D846del†	3.0	5.0	12400
2528_2539 del12	I843_S847>T	4.2	7.2	12407
2530_2541 del12	M844_S847del	3.2	6.2§	12402
2524_2532 del9	D842_M844del	1.5	4.5	12401
2524_2526 delGAC	D842del	0.9	3.9§	12406
2526_2538 >G¶	D842_D846>E	0.3	3.3§	12408
2524_2526 GAC>TAT	D842Y†	0.9	3.9§	12397

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

† The mutations 2524G>T and 2524_2526 GAC>TAT, and 2526_2537 del12 and 2527_2538 del12 result in the same amino acid change, respectively.

‡ The mutations 2524_2535del12 and 2526_2537del12 result in the same nucleic acid change.

§ The LOD for these deletions in *PDGFRA* exon 18 were determined by adding 3 standard deviations of blank measurements to the LOB value.

¶ The mutation 2526_2538 >G cannot be analyzed in the AQ mode of the PyroMark Q24 Software.

Linearity

Linearity was determined using mixtures of plasmids carrying the wild-type or mutant sequence for the duplication 1509_1510insGCCTAT in *KIT* exon 9 and the mutation 2525A>T in *PDGFRA* exon 18. The plasmids were mixed in proportions to give 4 levels of mutation (5, 10, 30, and 50%). Each mixture was analyzed with 3 different lots of the *therascreen* GIST RapidScreen Pyro Kit in 3 Pyrosequencing runs with 3 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 Figures 12 and 13.

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

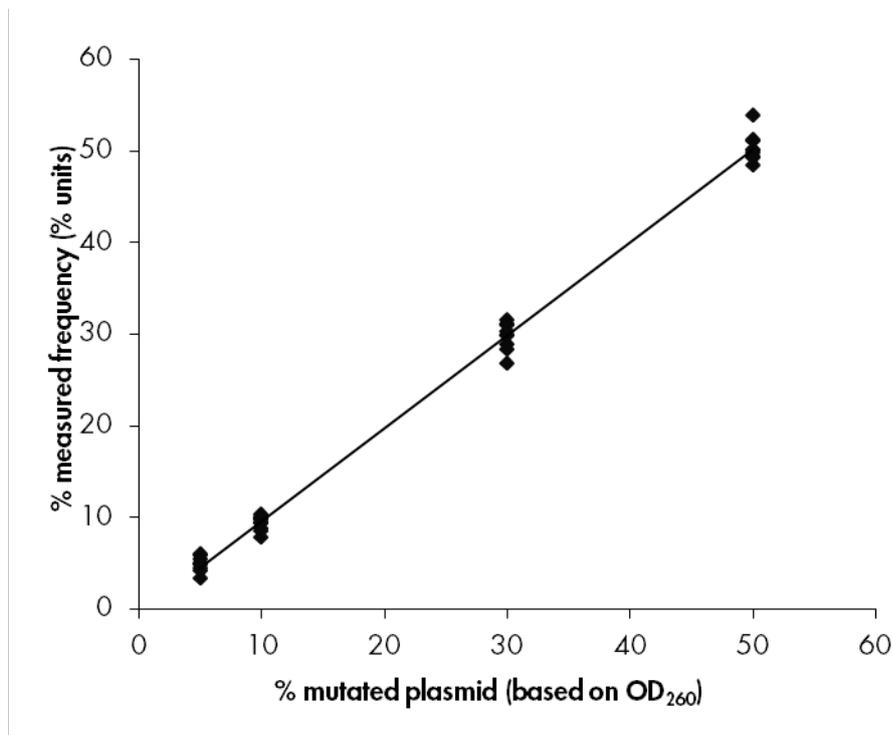


Figure 12. Linearity of the duplication 1509_1510insGCCTAT in *KIT* exon 9.

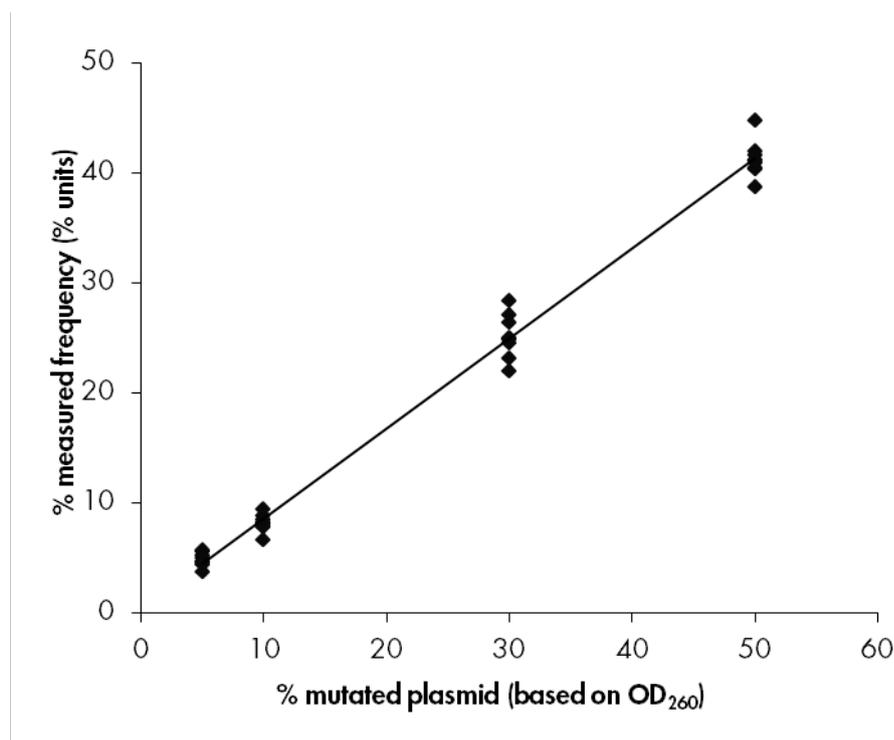


Figure 13. Linearity of the mutation 2525A>T in *PDGFRA* exon 18.

Precision

The precision data allows the determination of the total variability of the assays and was obtained at 3 different levels by analysis of the above mentioned plasmid mixtures with 3 replicates each.

Repeatability (intra-assay and inter-batch variability) was calculated based on the data for determination of linearity (3 runs on the same day using varying lots of the *therascreen* GIST RapidScreen Pyro Kit). Intermediate precision (intra-laboratory variability) was determined in 3 runs within one laboratory on 3 different days with varying operators, PyroMark Q24 instruments, and lots of the *therascreen* GIST RapidScreen Pyro Kit. Reproducibility (inter-laboratory variability) was calculated from 2 runs each in an internal and external laboratory and using varying lots of the *therascreen* GIST RapidScreen Pyro Kit.

Precision estimates are expressed as standard deviation of the measured mutation frequencies in % units (Table 11). The repeatability, intermediate precision, and reproducibility for the duplication 1509_1510insGCCTAT in *KIT* exon 9 was 0.8–1.6, 0.5–1.5, and 0.7–1.9 % units, respectively, in the measured range of 5 to 50% mutation level. The repeatability, intermediate precision, and reproducibility for the mutation 2525A>T in *PDGFRA* exon 18 was 0.6–1.9, 0.6–3.7, and 0.5–2.4 % units, respectively, in the measured range of 5 to 50% mutation level.

Table 11. Precision for the duplication 1509_1510insGCCTAT in *KIT* exon 9*

% mutated plasmid [†]	Repeatability		Intermediate precision		Reproducibility	
	Mean	SD	Mean	SD	Mean	SD
5	4.9	0.8	4.6	0.5	4.6	0.7
10	9.3	0.8	9.3	1.2	9.3	0.9
30	29.7	1.5	29.2	1.2	29.2	1.7
50	50.3	1.6	50.2	1.5	49.7	1.9

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

[†] Based on OD₂₆₀ measurement.

Table 12. Precision for the mutation 2525A>T in *PDGFRA* exon 18[‡]

% mutated plasmid [§]	Repeatability		Intermediate precision		Reproducibility	
	Mean	SD	Mean	SD	Mean	SD
5	4.8	0.6	4.8	0.6	5.0	0.5
10	8.2	0.8	7.7	0.7	8.8	1.0
30	25.1	1.9	23.6	3.4	26.6	1.4
50	41.2	1.6	40.5	3.7	43.3	2.4

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

[§] Based on OD₂₆₀ measurement.

Diagnostic evaluation

The *therascreen* GIST RapidScreen Pyro Kit was evaluated in comparison with Sanger sequencing. DNA was extracted from 100 formalin-fixed paraffin-embedded (FFPE) GIST tumor samples and analyzed for mutations in *KIT* exon 9 and *PDGFRA* exon 18.

DNA was isolated using the QIAamp DNA FFPE Tissue Kit. Analyses were carried out with the *therascreen* GIST RapidScreen Pyro Kit on the PyroMark Q24. Sanger sequencing was carried out on the Applied Biosystems[®] 3130 Genetic Analyzer.

Of 100 samples analyzed the mutational status could be determined for all *KIT* exon 9 (Figure 13) and *PDGFRA* exon 18 (Figure 14) with both methods.

Table 13. Results of the analyzed GIST tumor samples for *KIT* exon 9

<i>KIT</i> exon 9		Sanger sequencing		
		No mutation detected	1509_1510insGCCTAT Y503_F504insAY	Total
<i>therascreen</i> GIST RapidScreen Pyro Kit	No mutation detected	92	0	92
	1509_1510insGCCTAT Y503_F504insAY	0	8	8
	Total	92	8	100

Table 14. Results of the analyzed GIST tumor samples for *PDGFRA* exon 18

<i>PDGFRA</i> exon 18		Sanger sequencing				Total
		WT	2530-2541del12 M844_S847del	2526-2538>G D842_D846>E	2525A>T D842V	
<i>therascreen</i> GIST RapidScreen Pyro Kit	No Mutation detected	92	0	0	0	92
	2530-2541del12 M844_S847del	0	2	0	0	2
	2526-2538>G D842_D846>E	0	0	3	0	3
	2525A>T D842V	1	0	0	2	3
	Total	93	2	3	2	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. The Pyrosequencing data was analyzed using the GIST RapidScreen Plug-in Report.

References

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

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

Cited references

1. The ESMO/European Sarcoma Network Working Group (2012) Gastrointestinal stromal tumors: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann. Oncol.* **23** (Supplement 7), vii49.
2. Gastrointestinal Stromal Tumor Meta-Analysis Group (MetaGIST) (2010) Comparison of two doses of imatinib for the treatment of unresectable or metastatic gastrointestinal stromal tumors: A meta-analysis of 1,640 patients. *J. Clin. Oncol.* **28**, 1247.
3. Joensuu, H. (2006) Gastrointestinal stromal tumor (GIST). *Ann. Oncol.* **17** (Supplement 10), x280.

Symbols

The following symbols may appear on the packaging and labeling:



<N>

Contains reagents sufficient for <N> tests



Use by



In vitro diagnostic medical device



Catalog number

LOT	Lot number
MAT	Material number
COMP	Components
CONT	Contains
NUM	Number
GTIN	Global Trade Item Number
	Temperature limitation
	Manufacturer
	Consult instructions for use
	Caution

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Appendix A: Setting Up *therascreen* GIST RapidScreen Pyro Assays

If the GIST RapidScreen Plug-in Report has been installed, pre-defined Assay Setups for *KIT* exon 9 and *PDGFRA* exon 18 are available in the shortcut browser of the PyroMark Q24 Software under the path “Example Files/PyroMark Setups/GIST”. The following steps do not need to be performed. The GIST RapidScreen Plug-in Report can be obtained from pyro.plugin@qiagen.com.

We strongly recommend the use of the GIST RapidScreen Plug-in Report over manual analysis. Complex mutations of *PDGFRA* exon 18 cannot be added manually to a “Sequence to Analyze” and have to be analyzed using the GIST RapidScreen Plug-in Report. After installation of the plug-in or each time new software is installed or upgraded on the office computer, the correct function of the Plug-in should be verified as described in the GIST RapidScreen Plug-In Quick Guide.

If the GIST RapidScreen Plug-in Report has not been installed, the assay files must be set up manually before running the *therascreen* GIST RapidScreen Pyro assays for the first time. Set up the assay for *KIT* exon 9 and *PDGFRA* exon 18 by using the PyroMark Q24 Software, as described below.

Procedure

KIT exon 9

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

A2. Manually enter the following “Dispensation Order”:
CTCTGCTGACTTGC

A3. Type the following sequence in “Sequence to Analyze”:
TCTGCCTAT[GCCTAT]TTAA

The 6 bp duplication GCCTAT after codon 503 in *KIT* exon 9 will be detected using this “Sequence to Analyze”.

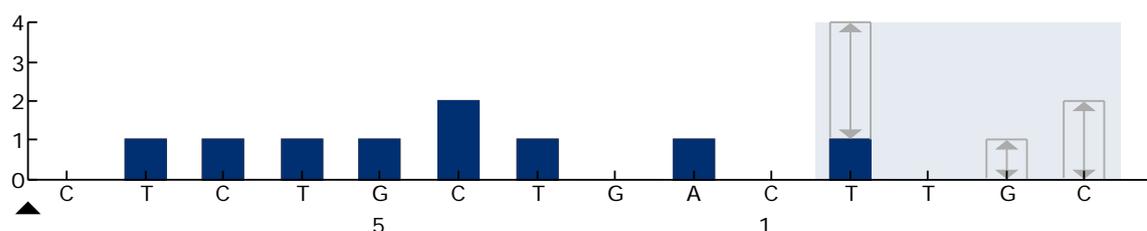


Figure 14. Histogram for *KIT* exon 9 with the “Sequence to Analyze” TCTGCCTAT[GCCTAT]TTAA addressing the 6 bp duplication after codon 503.

- 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 “KIT exon 9”.

PDGFRA exon 18

- A1. Click  in the toolbar and select “New AQ Assay”.
- A2. Manually add the following “Dispensation Order”:
GCAGAGTACTATCATGCATGATCGACTAG
- A3. Type the following sequence in “Sequence to Analyze”:
CCAGAGWCATCATGCATGATTCGAACTAT

The most frequent mutation GAC>GTC in codon 842 (nucleotide 2525) of PDGFRA exon 18 will be detected using this “Sequence to Analyze”.

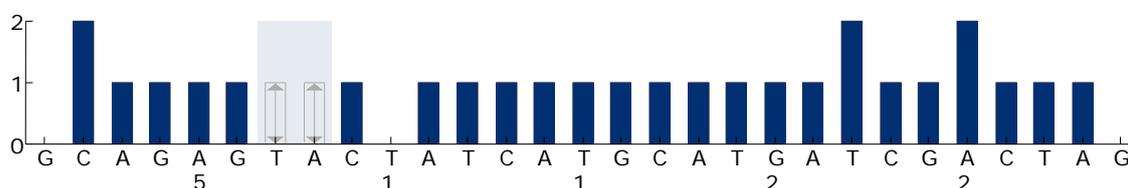


Figure 15. Histogram for PDGFRA exon 18 with the “Sequence to Analyze” CCAGAGWCATCATGCATGATTCGAACTAT addressing the mutation GAC>GTC in codon 842 (nucleotide 2525).

The “Sequence to Analyze” can be changed after the run to additionally analyze for mutations at nucleotide 2524 (codon 842) as well as 9 deletions and complex mutations within the region of codon 842 to 847.

To check if the following mutations are present, change the “Sequence to Analyze” according to Table 15.

Note: The warning “Quantification may be uncertain: the variable position requires more than 5 dispensations” during assay setup can be disregarded.

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

- 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 “PDGFRA exon 18”.

Table 15. Common mutations detected by the *therascreen* GIST RapidScreen Pyro Kit using different “Sequence to Analyze”

Nucleic acid change	Amino acid change	“Sequence to Analyze”
<i>KIT</i> exon 9		
1509_1510 insGCCTAT	Y503_F504insAY	TCTGCCTAT[GCCTAT] TTAA*
<i>PDGFRA</i> exon 18		
2525A>T	D842V	CCAGAGWCATCATGC ATGATTCGAACTAT*
2524G>T	D842Y [†]	CCAGAKACATCATGCAT GATTCTCGAACTAT
2524_2535 del12 or [‡] 2526_2537 del12	D842_H845del or [‡] I843_D846del [†]	CCAGAGA[CATCATGC ATGA]TTCGAACTAT
2527_2538 del12	I843_D846del [†]	CCAGAGAC[ATCATGC ATGAT]TCGAACTAT
2528_2539 del12	I843_S847>T	CCAGAGACA[TCATGC ATGATT]CGAACTAT
2530_2541 del12	M844_S847del	CCAGAGACATC [ATGCATGATTCG] AACTATGTGT
2524_2532 del9	D842_M844del	CCAGA[GACATCATG] CATGATTCTCGAACTAT
2524_2526 delGAC	D842del	CCAGA[GAC]ATCATG CATGATTCTCGAACTAT
2526_2538 >G	D842_D846>E	– [§]
2524_2526 GAC>TAT	D842Y [†]	– [§]

* Standard “Sequence to Analyze”.

[†] The mutations 2524G>T and 2524_2526 GAC>TAT, and 2526_2537 del12 and 2527_2538 del12 result in the same amino acid substitution, respectively.

[‡] The mutations 2524_2535del12 and 2526_2537del12 result in the same nucleic acid substitution and are analyzed by the same Sequence to Analyze.

[§] The mutation 2526_2538 >G and 2524_2526 GAC>TAT cannot be analyzed in the AQ mode of the PyroMark Q24 Software.

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.</p> <p>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 (Milli-Q 18.2 MΩ x cm, www.millipore.com, or equivalent).

Procedure

B1. Ensure that no vacuum is applied to the vacuum tool. Make sure that the vacuum is closed (Off) and the vacuum pump is switched off.

B2. Discard any solutions left in the troughs.

B3. Rinse the troughs with high-purity water, or replace them if necessary.

B4. Empty the waste container.

The cap can be removed without disconnecting the tubing.

B5. If the vacuum workstation must be cleaned (for example, due to dust or spillage), follow the instructions in the *PyroMark Q24 User Manual*.

Ordering Information

Product	Contents	Cat. no.
<i>therascreen</i> GIST RapidScreen 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	971510
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 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

* UK only.

† Rest of world.

Product	Contents	Cat. no.
Accessories		
PyroMark Q24 Plate (100)	24-well sequencing reaction plate	979301
PyroMark Q24 Cartridge (3)	Cartridges for dispensing nucleotides and reagents	979302
PyroMark Vacuum Prep Filter Probe (100)	Reusable filter probes for PyroMark Vacuum Workstation Q96 and Q24	979010
PyroMark Control Oligo	For installation check of system	979303
PyroMark Q24 Validation Oligo	For performance confirmation of system	979304
Related products		
QIAamp DNA FFPE Tissue Kit (50)	For 50 DNA preps: 50 QIAamp MinElute® Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	56404
EZ1 DNA Tissue Kit (48)	For 48 preps: Reagent Cartridges (Tissue), Disposable Filter-Tips, Disposable Tip-Holders, Sample Tubes (2 ml), Elution Tubes (1.5 ml), Buffer G2, Proteinase K	953034
QIAamp DSP DNA Blood Mini Kit	For 50 preps: QIAamp Mini Spin Columns, Buffers, Reagents, Tubes, VacConnectors	61104

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