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therascreen® UGT1A1 Pyro® Kit Handbook

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Version 1

IVD

For in vitro diagnostic use

CE

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Contents

Intended Use

The *therascreen* UGT1A1 Pyro Kit is an in vitro nucleic acid sequence-based detection test based on Pyrosequencing® technology for genotyping of the allele variants *28 and *6 of the human UGT1A1 gene in genomic DNA derived from human tissue samples.

The *therascreen* UGT1A1 Pyro Kit is intended to provide clinicians with information to aid the selection of patients with greater risk for decreased UDP-glucuronosyltransferase activity. 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.
- The PyroMark Q24 Software (version 2.0) and PyroMark Q24 MDx Software (version 2.0).

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

Summary and Explanation

The *therascreen* UGT1A1 Pyro Kit is used for genotyping of the allele variant *28 (for distinguishing between 6 and 7 TA repeats) and allele variant *6 (for distinguishing between G and A genotype) of the human UGT1A1 gene. The kit consists of two assays: one for genotyping of allele variant *28 and the second for genotyping of allele variant *6 (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 genotyping and quality assessment of the analysis.

The allele variant *28 is sequenced in reverse orientation and the allele variant *6 in forward orientation.

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 µl of each primer or primer mix.

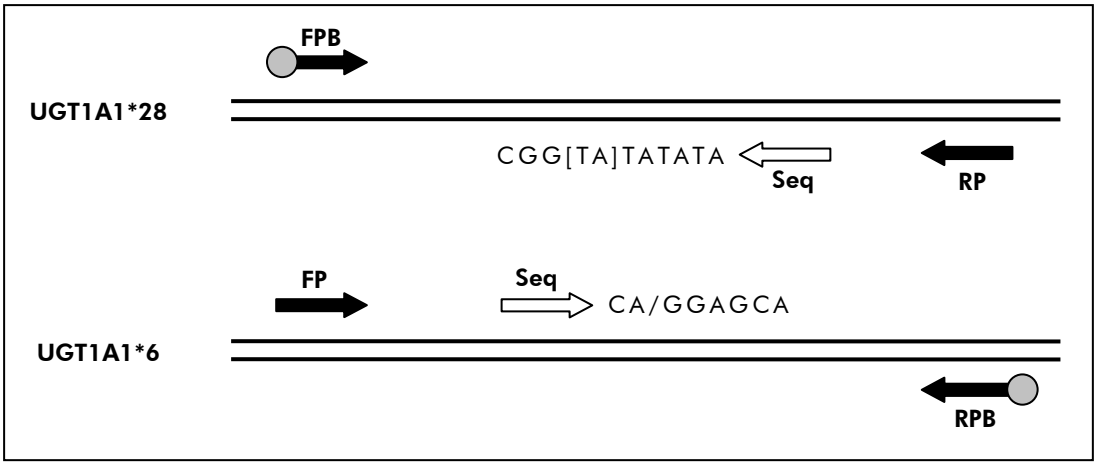


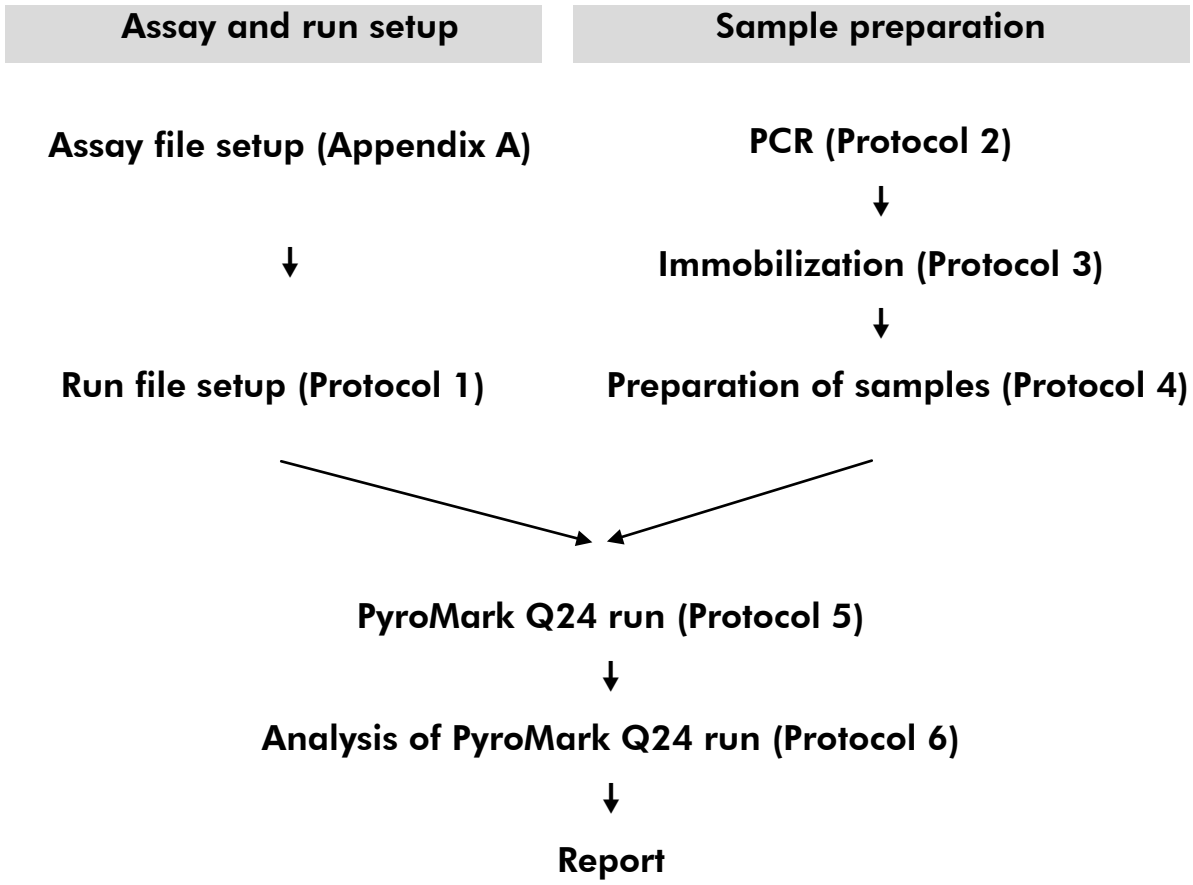
Figure 1. Illustration of the *therascreen* UGT1A1 assays. The sequence indicated is the analyzed sequence with polymorphic nucleotides indicated by square brackets or slash. Part of the TA repeats analyzed with the UGT1A1 *28 assay are covered by the sequencing primer. **FP, FPB**: Forward PCR primers (B indicates biotinylation); **RP, RPB**: Reverse PCR primers (B indicates biotinylation); **Seq**: Sequencing primers.

Principle of the Procedure

The workflow on page 7 illustrates the assay procedure. After PCR using primers targeting allele variants *28 and *6, the amplicons are immobilized on Streptavidin Sepharose® High Performance beads. Single-stranded DNA is prepared, and the corresponding sequencing primers anneal to the DNA. The samples are then analyzed on the PyroMark Q24 system using assay setup files and a run file.

Note: The workflow has been slightly modified compared to the *PyroMark Q24 User Manual* (see "Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24", page 23).

Workflow of *therascreen* UGT1A1 Pyro procedure



Controls

Human Control DNA is included in the kit as a positive control for PCR and sequencing reactions. This control DNA has a homozygous TA6/TA6 and G/G genotype when analyzed for the allele variants *28 and *6, respectively.

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

Materials Provided

Kit contents

therascreen UGT1A1 Pyro Kit (box 1/2)

***therascreen* UGT1A1 Pyro Kit**	**(24)**
Catalog no.	**971540**
Number of reactions	**24**
PCR Primer Mix UGT1A1 *28	24 µl
PCR Primer Mix UGT1A1 *6	24 µl
Seq Primer UGT1A1 *28	24 µl
Seq Primer UGT1A1 *6	24 µl
PyroMark PCR Master Mix, 2x	850 µl
CoralLoad® Concentrate, 10x	1.2 ml
H_2O	3 x 1.9 ml
Human Control DNA, 2 ng/µl	100 µl

therascreen buffers and reagents (box 2/2)

therascreen 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
dATPαS		1180 µl
dCTP		1180 µl
dGTP		1180 µl
dTTP		1180 µl
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 15)
- Pipets (adjustable)*
- Sterile pipet tips (with filters for PCR setup)
- Benchtop microcentrifuge*
- Thermal cycler* and appropriate PCR tubes
- Streptavidin Sepharose High Performance (GE Healthcare, cat. no. 17-5113-01; www.gelifesciences.com)
- PyroMark Q24 (cat. no. 9001514 or 9001513)*†
- PyroMark Q24 Software (cat. no. 9019062 or 9019063)†
- 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 (see "Recommended plate mixers", page 11)
- 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 product 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* UGT1A1 Pyro Kit.

Table 1. Plate mixers recommended for use with the *therascreen* UGT1A1 Pyro Kit

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

Warnings and Precautions

For In Vitro Diagnostic Use

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN® kit and kit component.

The following hazard and precautionary statements apply to components of the *therascreen* UGT1A1 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

The user should always pay attention to the following.

- Strict compliance with the user manual is required for optimal results. Dilution of the reagents, other than as described in this handbook, is not recommended and will result in a loss of performance.
- The workflow has been slightly modified (see "Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24", page 23) compared to the *PyroMark Q24 User Manual*.
- 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).

- 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 genotype.

Reagent Storage and Handling

The *therascreen* UGT1A1 Pyro Kit is shipped in two boxes. The *therascreen* UGT1A1 Pyro Kit (box 1/2) is shipped on dry ice. PyroMark PCR Master Mix, CoralLoad Concentrate, control DNA, and all primers should be stored at –30°C to –15°C upon arrival.

The Pyro buffers and reagents (box 2/2) containing buffers, Enzyme Mixture, Substrate Mixture, dATPαS, 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 6 freeze–thaw cycles.

Note: Nucleotides should not be frozen.

The *therascreen* UGT1A1 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 2 are recommended for DNA purification from the indicated human sample types for use with the *therascreen* UGT1A1 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* UGT1A1 Pyro Kit

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

* CE-IVD-marked in accordance with EU Directive 98/79/EC.

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

Protocol 1: Run setup for the PyroMark Q24 system

Things to do before starting

- Create an Assay Setup as described in "Appendix A: Setting Up *therascreen* UGT1A1 Pyro Assays", page 40. This needs to be done only once, before running the *therascreen* UGT1A1 Pyro assays for the first time.

Procedure

1. **Click in the toolbar.**
   A new run file is created.
2. **Enter the run parameters (see "Run parameters", page 17).**
3. **Set up the plate by adding assays for allele variant *28 and allele variant *6 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: A sample with Human Control DNA can be included for each assay as a positive control for the PCR and sequencing reactions (see "Controls", page 7).
4. **When the run is set up and ready to run on the PyroMark Q24, print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Select "Pre Run Information" from the "Tools" menu and, when the report appears, click .**
5. **Close the run file and copy it to a USB stick (supplied with the system) using Windows® Explorer.**
   The printed Pre Run Information can be used as a template for the sample set up (see "Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads", page 21).

   To run the plate on the 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.
Reagent ID:	**Optional**: Enter the lot numbers for the *therascreen* UGT1A1 Pyro Kit box 1 and box 2 to be used. The lot numbers can be found on the product label. **Note**: We recommend entering the lot numbers so that any unexpected problems with the *therascreen* UGT1A1 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 reagents supplied with the *therascreen* UGT1A1 Pyro Kit

This protocol is for PCR amplification of a region for genotyping of allele variant *28 and a separate PCR amplification of a region for genotyping of allele variant *6 using the *therascreen* UGT1A1 Pyro Kit.

Important points before starting

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

Things to do before starting

- Before opening the tubes with PCR primers, centrifuge briefly to collect contents at the bottom of the tubes.
- Adjust the concentration of the sample DNA to 0.4–2 ng/µl, if necessary. **Note**: The Human Control DNA included in the kit is provided at a concentration of 2 ng/µl.

Procedure

1. **Thaw all necessary components.**
   Mix well before use.
2. **Prepare a reaction mix for each PCR primer set according to Table 3.**
   The reaction mix typically contains all of the components needed for PCR except the sample.

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

Table 3. Preparation of reaction mix for each PCR primer mix

Component	Volume/reaction (µl)
PyroMark PCR Master Mix, 2x	12.5
CoralLoad Concentrate, 10x	2.5
PCR Primer mix UGT1A1 allele variant *28 **or** PCR Primer mix UGT1A1 allele variant *6	1.0
Water (H_2O, supplied)	4.0
Total volume	**20.0**

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

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

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

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

   Note: A sample with Human Control DNA can be included for each assay as a positive control for the PCR and sequencing reactions (see "Controls", page 7).

Table 4. Preparation of PCR

Component	Volume/reaction (µl)
Reaction mix	20
Sample DNA	5
Total volume	**25**

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

Table 5. Optimized cycling protocol

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

6. **Place the PCR tubes in the thermal cycler and start the cycling program.**
7. **After amplification, proceed with "Protocol 3: Immobilization of PCR products to Streptavidin Sepharose High Performance beads", page 21.**

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_2O, supplied)	28
Total volume	**70**

3. **Add 70 µl of the master mix to wells of a 24-well PCR plate (or strips) as predefined in the run setup (see "Protocol 1: Run setup for the PyroMark Q24 system", page 16).**
4. **Add 10 µl of biotinylated PCR product from Protocol 2 to each well containing master mix as predefined in the run setup (see "Protocol 2: PCR using the reagents supplied with the *therascreen* UGT1A1 Pyro Kit", page 18).**

   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.**

   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, 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 23.**

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

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

Protocol 4: Preparation of samples prior to Pyrosequencing analysis on the PyroMark Q24

This protocol is for preparation of single-stranded DNA and annealing of the sequencing primer to the template prior to Pyrosequencing analysis on the PyroMark Q24.

Important points before starting

- Before opening the tubes with sequencing primers, centrifuge briefly to collect contents at the bottom of the tubes.
- Add the 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 (allele variant *28 or allele variant *6).
- The workflow has been slightly modified compared to the *PyroMark Q24 User Manual* (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, add high-purity water to 25 ml 10x PyroMark Wash Buffer to achieve a final volume of 250 ml and obtain a 1x working solution.

  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 UGT1A1 *28 and Seq Primer UGT1A1 *6, 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 of dilution of the sequencing primers

Component	Volume/sample (µl)	Volume for 9 + 1 reactions (µl)
Seq Primer UGT1A1 *28 **or** Seq Primer UGT1A1 *6	0.8	8.0
PyroMark Annealing Buffer	24.2	242.0
Total volume	**25.0**	**250.0**

2. **Add 25 µl 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. **Place the PCR plate (or strips) from Protocol 3 and the PyroMark Q24 Plate on the worktable (see Figure 2).**

   Ensure that the plate is in the same orientation as when samples were loaded.

Figure 2. Placement of PCR plate (or strips) and PyroMark Q24 Plate on the vacuum workstation.

4. **Apply vacuum to the vacuum tool by opening the vacuum switch.**

5. **Carefully lower the filter probes 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 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).**

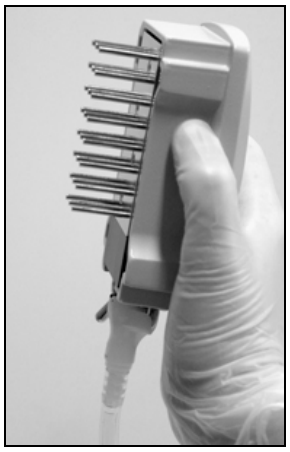


Figure 3. Illustration of the vacuum tool raised to beyond 90° vertical.

10. **While the vacuum tool is held over the PyroMark Q24 Plate, close the vaccum 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.**

    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 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 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: Emptying the Waste Container and Troughs", page 41).
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 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 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 16), 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 µl each of water (H_2O, supplied).**
2. **Mix by swirling the vial gently. Do not vortex!**

   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.

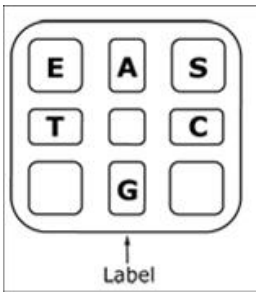


Figure 4. Illustration of the PyroMark Q24 Cartridge, as seen from above. The annotations correspond to the label on the reagent vials. Add enzyme mixture (**E**), substrate mixture (**S**), and nucleotides (**A**, **T**, **C**, **G**) according to the volume information given in the Pre Run information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

    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 genotyping analysis of a finished *therascreen* UGT1A1 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 AQ mode of PyroMark Q24 Software either by selecting "Open" in the "File" menu or by double-clicking the file ( ) in the shortcut browser.**
4. **To analyze the run and get an overview of the results, click one of the Analyze buttons.**

    Analyze all wells.

    Analyze the selected well.

   For more details on how to analyze a run, see the *PyroMark Q24 User Manual*.

5. **To generate a report, select "SNP Full Report" or "SNP Overview Report" 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* UGT1A1 Pyro Assays", page 40, and the *PyroMark Q24 User Manual*).

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

Interpretation of Results

The included Human Control DNA can be used for comparison of results. This control DNA has a homozygous TA6/TA6 and G/G genotype when analyzed for allele variants *28 and *6, respectively.

Genotyping analysis is automatically performed by the PyroMark Q24 Software and is provided in the “SNP Full Report” and “SNP Overview Report”.

Note: The quality assessment and warnings generated in the SNP reports are relevant for genotyping analysis. Additional quality assessments and warnings generated in the AQ mode of the PyroMark Q24 Software can be disregarded.

Representative results

Representative Pyrogram results are shown in Figures 5–10.

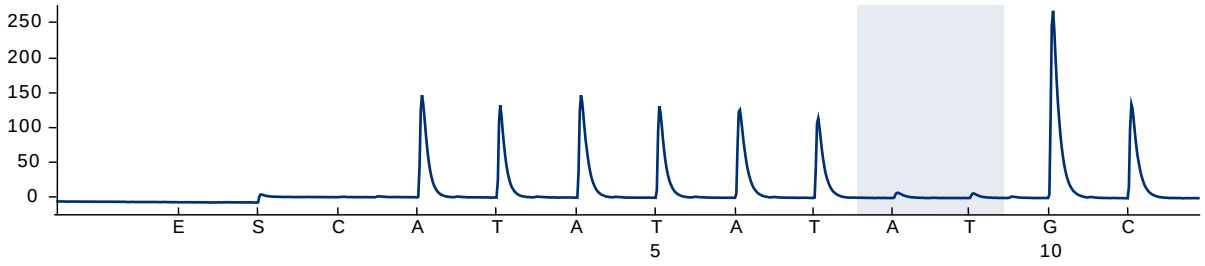


Figure 5. Pyrogram trace obtained after analysis of a sample with –/– (TA6/TA6) genotype when analyzed for allele variant *28.

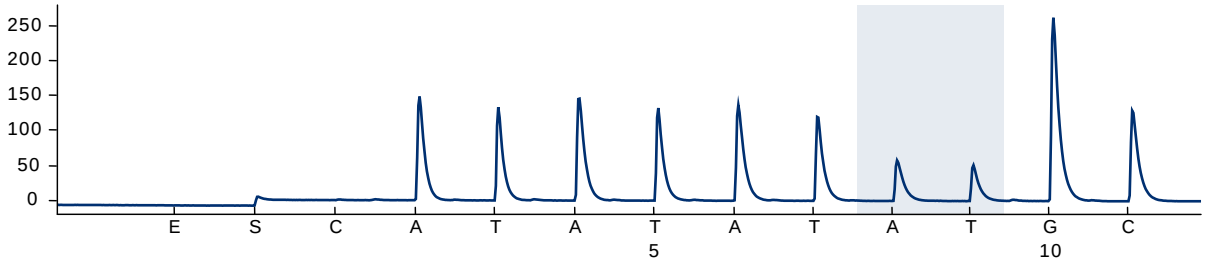


Figure 6. Pyrogram trace obtained after analysis of a sample with –/TA (TA6/TA7) genotype when analyzed for allele variant *28.

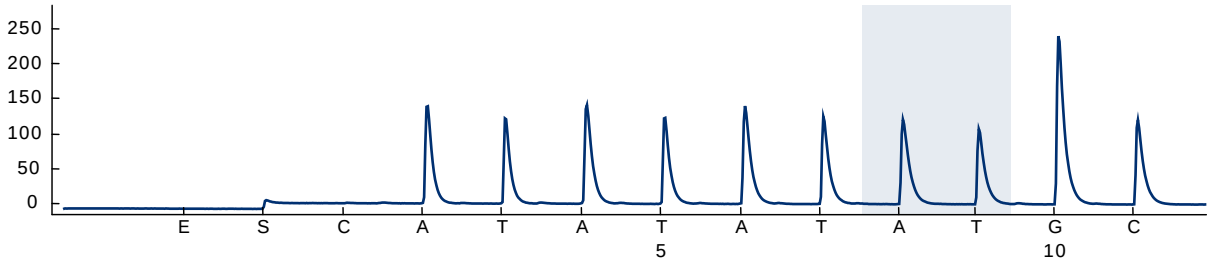


Figure 7. Pyrogram trace obtained after analysis of a sample with TA/TA (TA7/TA7) genotype when analyzed for allele variant *28.

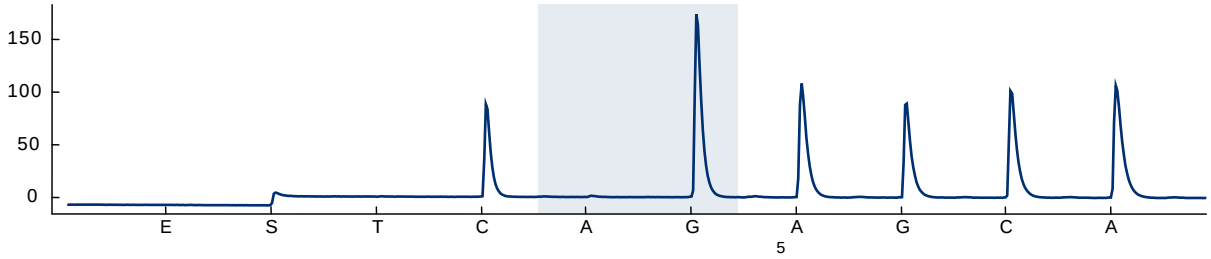


Figure 8. Pyrogram trace obtained after analysis of samples with a G/G genotype when analyzed for allele variant *6.

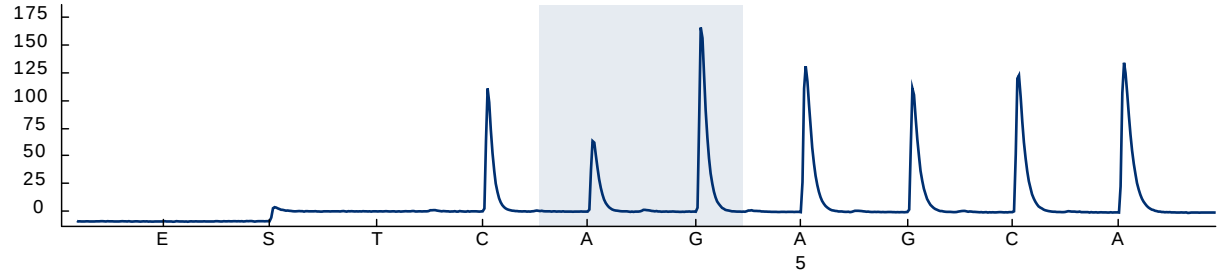


Figure 9. Pyrogram trace obtained after analysis of samples with a G/A genotype when analyzed for allele variant *6.

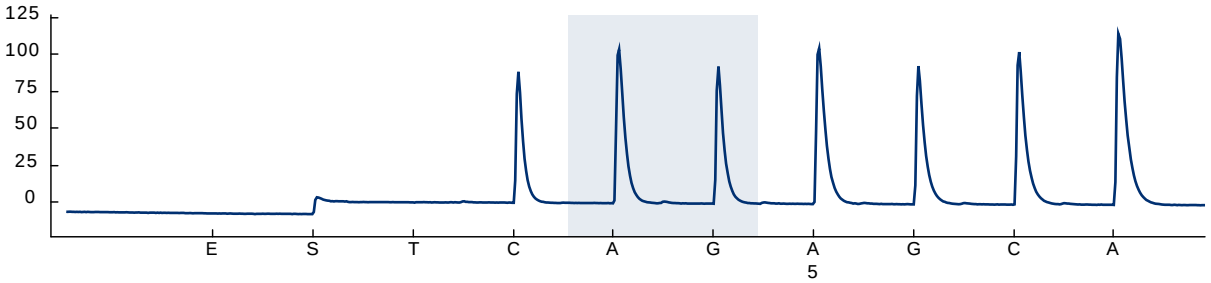


Figure 10. Pyrogram trace obtained after analysis of samples with an A/A genotype when analyzed for allele variant *6.

Troubleshooting guide

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

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

	Comments and suggestions
Signals in the no template control (negative control)	
a) Cross-talk between wells	Signal from one well is detected in a neighboring well. Avoid placing samples with high signal intensities next to no template control wells.
b) PCR contamination	Use sterile pipet tips with filters. Store and extract materials such as specimens, controls, and amplicons separately from PCR reagents.
Poor or unexpected sequence	
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 in SNP Report	
a) "Uncertain / Failed due to low peak height" warning	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 User Manual* on a regular basis and exchange filter probes when indicated. In case of a "Check" quality assessment, 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) "Uncertain / Failed genotype determination" warning	In case of a "Check" quality assessment, 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. For the UGT1A1 *28 assay, the warning can be caused by the slippage of the polymerase across TA repeats, which could be more pronounced for FFPE tumor samples. Make sure that high quality DNA is used as template (e.g., isolated from blood samples) or increase the template DNA amount.

	Comments and suggestions
c) Unexpected rare allele variants	A "Check" or "Failed" quality assessment can be caused by an unexpected pattern of peaks. This might indicate an unexpected allele variant, which is not analyzed by the provided "Sequence to Analyze". These samples should be analyzed using alternative "Sequence to Analyze" considering unexpected allele variants.
d) High peak height deviation warning at dispensation x	The Pyrogram should be carefully compared to the histogram, which can be displayed by a right-click in the Pyrogram window. In case the measured peaks do not match the height of the histogram bars and cannot be explained by rare allele variants, it is recommended to rerun the sample.
High background	
a) Incorrect storage of nucleotides	Store nucleotides at 2–8°C. Storage at –15 to –25°C can cause an increase in the background.
b) Short cooling time of samples prior to Pyrosequencing analysis	Keep the samples on a PyroMark Q24 plate holder at room temperature for 10–15 minutes. Do not shorten the cooling time.
c) Contamination of cartridge	Carefully clean the cartridge as described in the product sheet. Store the cartridge protected from light and dust.
No signals in positive controls	
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 *therascreen* 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 User 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* UGT1A1 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

Precision

The precision data allows the determination of the total variability of the assay in regard to the correct genotyping of *28 and *6 allele variants. Plasmids carrying the allele variants were mixed in proportions (0, 50, 100%) representing the homo- and heterozygous genotypes (*28 TA6/TA6, TA6/TA7, and TA7/TA7; *6 G/G, G/A, and A/A). Each mixture was analyzed in seven Pyrosequencing runs with three replicates each with varying lots of the *therascreen* UGT1A1 Pyro Kit, PyroMark Q24 instruments, operators, days, and laboratories.

Precision is expressed as the Correct Call Rate (i.e., the proportion of analyzed samples with a correct genotyping result). The assays for genotyping analysis of the *28 and *6 allele variants listed in Table 8 and 9, respectively, showed a Correct Call Rate of 100% for the samples analyzed.

Table 8. Precision for genotyping of *28 allele variants

Genotype*	Number of samples	Correct calls
Homozygous TA6/TA6	21	21
Heterozygous TA6/TA7	21	21
Homozygous TA7/TA7	20	20

* Represented by 0, 50, and 100% plasmid mixtures based on OD_{260} measurement.

Table 9. Precision for genotyping of *6 allele variants

Genotype*	Number of samples	Correct calls
Homozygous G/G	21	21
Heterozygous G/A	21	21
Homozygous A/A	21	21

* Represented by 0, 50, and 100% plasmid mixtures based on OD_{260} measurement.

Diagnostic evaluation

The *therascreen* UGT1A1 Pyro Kit was evaluated in comparison with Sanger sequencing. DNA was extracted from 100 formalin-fixed paraffin-embedded (FFPE) tumor samples and analyzed for *28 and *6 allele variants.

DNA was isolated using the QIAamp DNA FFPE Tissue Kit. Pyrosequencing analysis was carried out with the *therascreen* UGT1A1 Pyro Kit on the PyroMark Q24 and Sanger sequencing on the ABI™ 3130 Genetic Analyzer.

Of 100 samples analyzed by Sanger sequencing, the genotype could be determined in 95 and 99 samples for the *28 and *6 allele variants, respectively. With the *therascreen* UGT1A1 Pyro Kit, it was possible to determine the genotype in 98 and 99 samples for the *28 and *6 allele variants, respectively.

Twenty nine, 49, and 12 samples were reported by both methods to have a TA6/TA6, TA6/TA7, and TA7/TA7 genotype, respectively. Four additional samples showed a TA6/TA6 genotype using the *therascreen* UGT1A1 Pyro Kit while Sanger sequencing detected a TA6/TA7 genotype (Table 10).

Excluding samples that failed in one or both methods, the *therascreen* UGT1A1 Pyro Kit and Sanger sequencing showed 96% concordance in results for genotyping of *28 allele variants (Table 10).

Table 10. Genotyping results for the *28 allele variants in samples of Caucasian origin

		Sanger sequencing				
		TA6/ TA6	**TA6/ TA7**	**TA7/ TA7**	**Unknown**	**Total**
***therascreen* UGT1A1 Pyro Kit**	**TA6/TA6**	29	4	0	2	**35**
	TA6/TA7	0	49	0	2	**51**
	TA7/TA7	0	0	12	0	**12**
	Unknown	0	1	0	1	**2**
	Total	**29**	**54**	**12**	**5**	**100**

All samples showed a homozygous G/G genotype for the *6 allele variant using both Sanger sequencing and the *therascreen* UGT1A1 Pyro Kit. This result is in line with state-of-the-art knowledge that the A/G and A/A genotypes are virtually absent from Caucasian populations. Therefore, DNA from additional 26 buccal swab samples collected from Asian people was isolated using the QIAamp DSP DNA Blood Mini Kit on the QIAcube® and analyzed for *6 allele variants.

Fifteen, nine, and two samples were reported by both methods to have a G/G, G/A, and A/A genotype, respectively (Table 11).

Excluding samples that failed in one or both methods, the *therascreen* UGT1A1 Pyro Kit and Sanger sequencing showed 100% concordance in results for *6 allele variants (Table 11).

Table 11. Genotyping results for the *6 allele variants in samples derived from Asian origin

		Sanger sequencing				
		G/G	**G/A**	**A/A**	**Unknown**	**Total**
***therascreen* UGT1A1 Pyro Kit**	**G/G**	15	0	0	0	**15**
	G/A	0	9	0	0	**9**
	A/A	0	0	2	0	**2**
	Unknown	0	0	0	0	**0**
	Total	**15**	**9**	**2**	**0**	**26**

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 blood or formalin-fixed paraffin-embedded tissue.

References

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

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

Symbols

Contains reagents sufficient for <N> tests


Use by

In vitro diagnostic medical device

Catalog number

Lot number

Material number

Components

Contains

Number

Sodium hydroxide

Global Trade Item Number


Temperature limitation


Manufacturer

Consult instructions for use

Contact Information

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Appendix A: Setting Up *therascreen* UGT1A1 Pyro Assays

Before running the *therascreen* UGT1A1 assay for the first time, the assay file must be set up. Set up the assay for UGT1A1 allele variants by using the PyroMark Q24 Software, as described below.

Procedure

UGT1A1 *28

1. **Click in the toolbar and select "New AQ Assay".**
2. **Type the following sequence in "Sequence to Analyze". *ATATAT[AT]GGCA***
3. **Manually enter the following "Dispensation Order". *CATATATATGC***
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 *UGT1A1 *28*.**

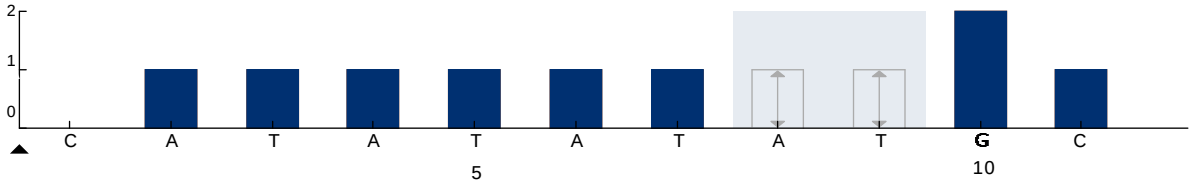


Figure 11. Histogram for genotyping of UGT1A1 allele variant *28.

UGT1A1 *6

1. **Click in the toolbar and select "New AQ Assay".**
2. **Type the following sequence in "Sequence to Analyze". *CRGAGCAT***
3. **Manually add the following "Dispensation Order". *TCAGAGCA***
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 *UGT1A1 *6*.**

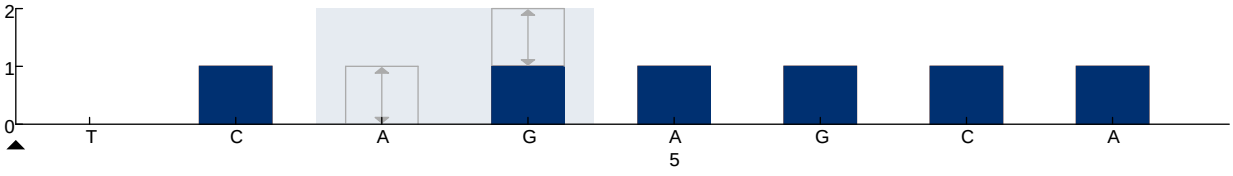


Figure 12. Histogram genotyping of UGT1A1 allele variant *6.

Appendix B: Emptying the Waste Container and Troughs

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

* OSHA: Occupational Safety and Health Administration (United States of America)

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

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

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

Important point before starting

- This protocol requires high-purity water.

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.
therascreen UGT1A1 Pyro Kit (24)	For 24 reactions on PyroMark Q24 Systems: Seq Primers, PCR Primers, Human Control DNA, PyroMark PCR Master Mix, CoralLoad Concentrate, PyroMark Binding Buffer, PyroMark Annealing Buffer, PyroMark Denaturation Solution, PyroMark Wash Buffer, Enzyme Mixture, Substrate Mixture, dATPαS, dCTP, dGTP, dTTP, and H_2O	971540
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		
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

Product	Contents	Cat. no.
PyroMark Q24 MDx Vacuum Workstation	Vacuum Workstation (220 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001517* 9001515†
PyroMark Q24 Vacuum Workstation	Vacuum Workstation (220 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001518
PyroMark Q24 MDx Software	Application software	9019063
PyroMark Q24 Software	Analysis software	9019062
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

* UK only

† Rest of world

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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