November 2010

PyroMark® HFE Handbook

For genotyping of the H63D and S65C variants in exon 2 and C282Y variant in exon 4 of the human HFE gene

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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- Nucleic acid and protein assays
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- Automation of sample and assay technologies

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Contents

Kit Contents

PyroMark HFE	
Catalog no.	**972442**
Number of preps:	
PyroMark Q24	**8 x 24**
PyroMark Q96 ID	**1 x 96**
PyroMark Q96 MD	**2 x 96**
Forward Exon 2 HFE PCR Primer	1 vial
Reverse Exon 2 HFE PCR Primer	1 vial
Forward Exon 4 HFE PCR Primer	1 vial
Reverse Exon 4 HFE PCR Primer	1 vial
Exon 2 HFE Sequencing Primer	1 vial
Exon 4 HFE Sequencing Primer	1 vial
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Shipping and Storage

PyroMark HFE is shipped on dry ice. PyroMark HFE should be stored at –15 to –25°C upon arrival. Dissolved primers should be stored at –15 to –25°C.

Product Use Limitations

PyroMark HFE is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

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Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding PyroMark HFE, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

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).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of PyroMark HFE is tested against predetermined specifications to ensure consistent product quality.

Safety Information

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

CAUTION: 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 Material Safety Data Sheets (MSDSs) 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).

24-hour emergency information

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Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

These assays are for genotyping the SNPs H63D and S65C in exon 2 and SNP C282Y in exon 4 of the human hereditary hemochromatosis (HFE) gene.

Principle and procedure

Two fragments spanning the polymorphisms are amplified by a duplex PCR and two Pyrosequencing® single nucleotide polymorphism (SNP) analyses are performed (Figure 1).

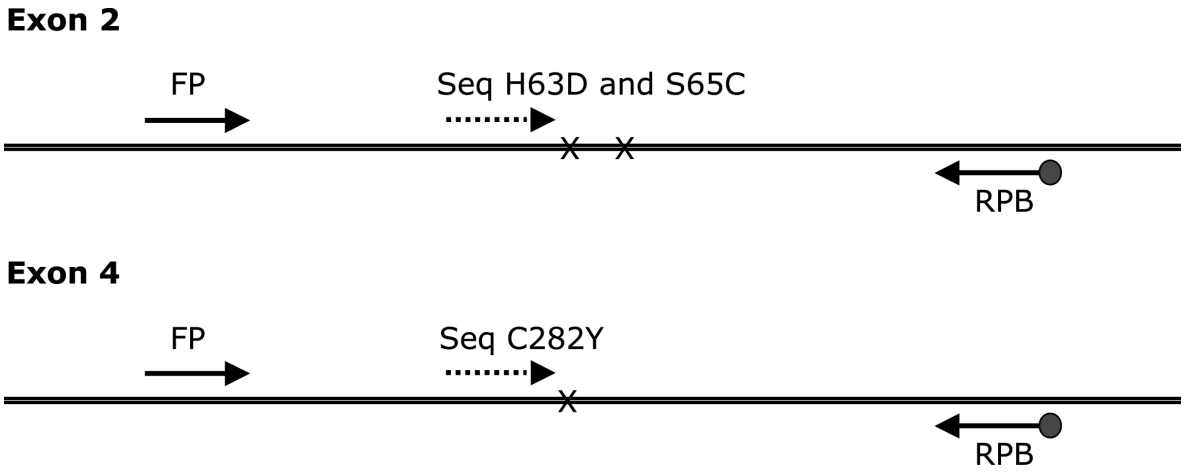


Figure 1. PyroMark HFE assays. The analyzed SNPs are shown. PCR primers are shown as solid arrows, while sequencing primers are shown as dashed arrows. **FP**: Forward primer; **RPB**: Reverse biotinylated primer; **Seq**: Sequencing primer.

Description of protocols

This handbook provides all necessary information for Pyrosequencing analysis of HFE and is organized into sets of protocols specific for the PyroMark Q24, PyroMark Q96 ID, and PyroMark Q96 MD.

The workflow for all three instruments begins with a common PCR amplification, regardless of instrument. Therefore, begin with the protocol "PCR Using PyroMark PCR Kit" (page 13). For the subsequent steps, use the protocols specific for the instrument you are using.

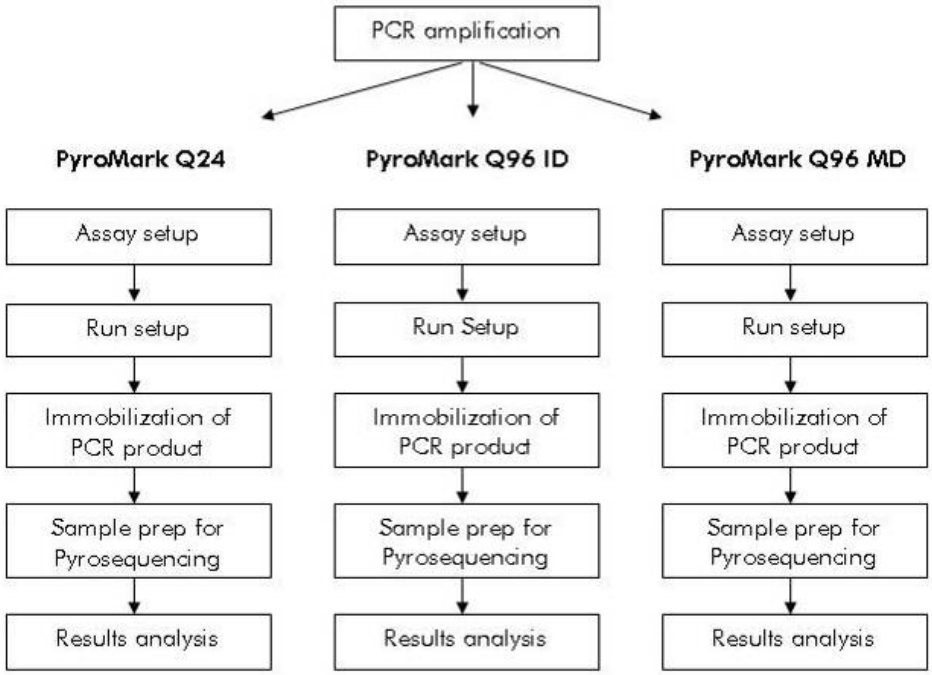


Figure 2. PyroMark HFE workflow.

Protocols for the PyroMark Q24

If using the PyroMark Q24, follow the instructions in protocol "Assay and Run Setup Using PyroMark Q24 Software" (page 16). Note that you only need to set up the HFE Assay the first time the PyroMark HFE is used, but a new Run must be set up each time the assay is performed. After amplification, follow the protocols "Immobilization of PCR Products to Streptavidin Sepharose® HP Beads for Analysis Using the PyroMark Q24" (page 18) and "Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q24" (page 20) to generate the sequencing templates. Finally, follow the protocol "Protocol: Genotyping of the HFE Gene Using the PyroMark Q24" (page 23) to perform the Pyrosequencing run and analyze the data.

Protocols for the PyroMark Q96 ID

If using the PyroMark Q96 ID, follow the instructions in protocol "Entry and Run Setup Using PyroMark Q96 ID Software" (page 16). Note that you only need to set up the HFE Entry the first time the PyroMark HFE is used, but a new Run must be set up each time the assay is performed. After amplification, follow the protocols "Immobilization of PCR Products to Streptavidin Sepharose HP Beads for Analysis Using the PyroMark Q96 ID" (page 29) and "Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q96 ID" (page 31) to generate the sequencing templates. Finally, follow the protocol "Protocol: Genotyping of Exons 2 or 4 of HFE Using the PyroMark Q96 ID" (page 34) to perform the Pyrosequencing run and analyze the data.

Protocols for the PyroMark Q96 MD

If using the PyroMark Q96 MD, follow the instructions in protocol “Entry and Run Setup Using PyroMark Q96 MD Software” (page 37). Note that you only need to set up the HFE Entry the first time the PyroMark HFE is used, but a new Run must be set up each time the assay is performed. After amplification, follow the protocols “Immobilization of PCR Products to Streptavidin Sepharose HP Beads for Analysis Using the PyroMark Q96 MD” (page 40) and “Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q96 MD” (page 42) to generate the sequencing templates. Finally, follow the protocol “Protocol: Genotyping of Exons 2 and 4 of HFE Using the PyroMark Q96 MD” (page 45) to perform the Pyrosequencing run and analyze the data.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

For use with PyroMark Q24

- PyroMark Q24 (cat. no. 9001514)
- PyroMark Q24 Software (cat. no. 9019062)
- PyroMark Q24 Plate (100) (cat. no. 979201)
- PyroMark Gold Reagents (5 x 24) (cat. no. 970802)
- PyroMark Q24 Cartridge (3) (cat. no. 979202)
- PyroMark Q24 Vacuum Workstation (cat. no. varies depending on region, see Ordering Information, page 54)

For use with PyroMark Q96 ID

- PyroMark Q96 ID (cat. no. 9001525)
- PyroMark Q96 ID Software (cat. no. 9019083)
- PyroMark Q96 Plate Low (100) (cat. no. 979002)
- PyroMark Q96 Sample Prep Thermoplate Low (cat. no. 9019070)
- PyroMark Q96 Cartridge (3) (cat. no. 979004)
- PyroMark Gold Q96 Reagents (5 x 96) (cat. no. 972804)
- PyroMark Q96 Vacuum Workstation (cat. no. varies depending on region, see Ordering Information, page 54)

For use with PyroMark Q96 MD

- PyroMark Q96 MD (cat. no. 9001526)
- PyroMark Q96 MD Software (cat. no. 9019085)
- PyroMark Q96 HS Plate (100) (cat. no. 979101)
- PyroMark Q96 HS Sample Prep Thermoplate (cat. no. 9019071)
- PyroMark Q96 HS Capillary Tip Holder (cat. no. 9019076) or PyroMark Q96 HS Dispensing Tip Holder (cat. no. 9019075)
- PyroMark Q96 HS Reagent Tips (4) (cat. no. 979102)
- PyroMark Q96 HS Capillary Tips (8) (cat. no. 979104) or PyroMark Q96 HS Nucleotide Tips (8) (cat. no. 979103)

- PyroMark Gold Q96 CDT Reagents (6 x 96) (cat. no. 972824) or PyroMark Gold Q96 Reagents (5 x 96) (cat. no. 972804)
- PyroMark Q96 Vacuum Workstation (cat. no. varies depending on region, see Ordering Information, page 54)

Additional equipment and reagents required

- PyroMark PCR Kit (cat. no. 978703)
- PyroMark Binding Buffer (cat. no. 979006)
- PyroMark Denaturation Solution (cat. no. 979007)
- PyroMark Wash Buffer, concentrate (cat. no. 979008)
- PyroMark Annealing Buffer (cat. no. 979009)
- High-purity water (Milli-Q® 18.2 MΩ x cm or equivalent)
- Ethanol (70%)
- Streptavidin Sepharose High Performance (GE Healthcare, cat. no. 17-5113-01; www.gelifesciences.com)
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80°C
- 96-well PCR plate or strips
- Strip caps
- Pipets (adjustable)
- Sterile pipet tips (with filters for PCR setup)

Protocol: PCR Using the PyroMark PCR Kit

Important points before starting

- The amplification of the two fragments spanning the polymorphisms is performed in a single reaction by a duplex PCR.
- For more detailed information, see the *PyroMark PCR Kit Handbook*.
- HotStarTaq® DNA Polymerase requires an activation step of **15 min at 95°C** (step 6 of the protocol).
- Set up all reaction mixtures in an area separate from that used for DNA preparation or PCR product analysis.
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.
- Before opening the tubes containing PCR primers, spin briefly to collect contents at the bottom of the tubes.
- Dissolve each PCR primer in 120 µl high-purity water (Milli-Q 18.2MΩ x cm or equivalent, filtered through 0.22 µm filter).

Procedure

1. **Thaw the PyroMark PCR Master Mix, CoralLoad® Concentrate, primer solutions.**

   It is important to mix the solutions before use in order to avoid localized concentrations of salt.

2. **Set up the reaction according to Table 1.**

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

3. **Gently pipet the Master Mix up and down for thorough mixing and dispense appropriate volumes into PCR tubes.**
4. **Add 10 ng human genomic DNA to the individual PCR tubes.**

   If using a thermal cycler without a heated lid, overlay with approximately 100 µl mineral oil.

5. **Program the thermal cycler according to Table 2.**

Table 1. Reaction composition using PyroMark PCR Master Mix

Component	Volume/reaction	Final concentration
PyroMark PCR Master Mix, 2x	12.5 µl	Contains HotStarTaq DNA Polymerase,1x PyroMark PCR Buffer,* and dNTPs
CoralLoad Concentrate, 10x	2.5 µl	1x
Forward Exon 2 and Exon 4 HFE Primer	0.5 µl each	0.2 µM
Reverse Exon 2 and Exon 4 HFE Primer	0.5 µl each	0.2 µM
RNase-free water	Variable	–
Template DNA, added at step 4	Variable	10 ng human gDNA
Total volume	**25 µl**	

* Contains 3 mM $MgCl_2$ (final concentration of 1.5 mM)

Table 2. Optimized cycling protocol for PyroMark PCR Master Mix

			Additional comments
Initial PCR activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling:			
Denaturation	30 s	94°C	
Annealing	30 s	60°C	
Extension	30 s	72°C	
Number of cycles	45		
Final extension	10 min	72°C	
Hold	∞	4°C	

6. **Place the PCR tubes in the thermal cycler and start the cycling program.**

   Note: After amplification, samples can be stored overnight at 2–8°C or at –20°C for longer storage.

7. **See Table 3 for the amounts of PCR product per sample required for subsequent Pyrosequencing analysis.**
8. **Proceed to the protocols corresponding to the instrument in use:**

9a. If using the PyroMark Q24, proceed to protocol "Entry and Run Setup Using PyroMark Q24 Software", page 16.

9b. If using the PyroMark Q96 ID, proceed to protocol "Entry and Run Setup Using PyroMark Q96 ID Software", page 26.

9c. If using the PyroMark Q96 MD, proceed to protocol "Entry and Run Setup Using PyroMark Q96 MD Software", page 37.

Table 3. Amounts of PCR product required for Pyrosequencing analysis

Instrument	PCR product required
PyroMark Q24	5–10 µl
PyroMark Q96 ID	10 µl
PyroMark Q96 MD	5–10 µl

Protocol: Assay and Run Setup Using PyroMark Q24 Software

This protocol is for setting up the Assay parameters and creating a Run Setup for analysis of SNPs H63D and S65C in exon 2 and SNP C282Y in exon 4 of the HFE gene using the PyroMark Q24.

The two exons each require a different Assay Setup. This protocol includes instructions for setting up the two Assays.

Important points before starting

- For further information on how to create a Run Setup, see the *PyroMark Q24 Software Online Help*.
- Assays for exon 2 and exon 4 are performed in separate wells, but can be run on the same plate.
- Steps1–6 are only performed the first time the assay is run.

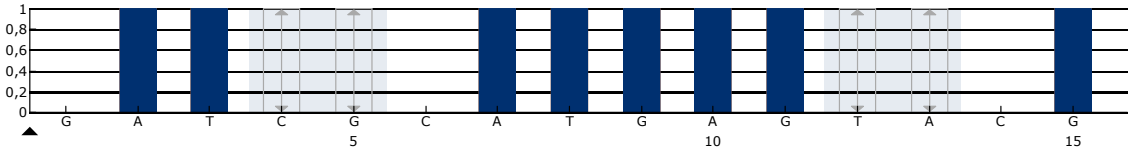
Procedure

1. **Set up the assay for exon 2 using the PyroMark Q24 Software. Select "New AQ Assay" and enter the following sequence in "Sequence to Analyze":**

   ATC/GATGAGT/AGT

2. **Manually enter the following Dispensation order:**

   GATCGCATGAGTACG



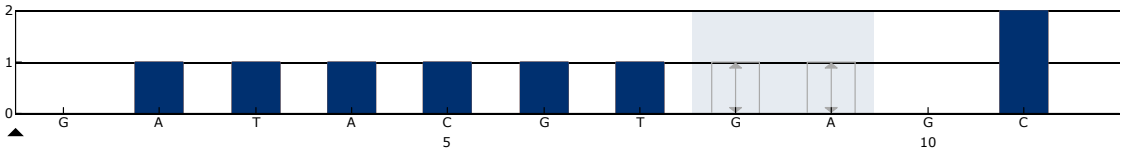
Histogram for the H63D and S65C variants in HFE Exon 2.

3. **Save the assay as "HFE_exon2".**
4. **Set up the assay for Exon 4 C282Y using the PyroMark Q24 Software. Select "New AQ Assay" and enter the following sequence in "Sequence to Analyze":**

   ATACGTG/ACCA

5. **Manually enter the following Dispensation order:**

   GATACGTGAGC



Histogram for the C282Y variant in HFE Exon 4.

6. **Save the assay as "HFE_exon4".**
7. **Define the Assays and Instrument parameters (see the *PyroMark Q24 User Manual* for more information).**
8. **Create a run file by selecting "New Run". Set up the plate by adding the assays for exons 2 and 4 to the number of wells to be analyzed.**

   Note: Be sure to set up one well for analysis of exon 2 and another for exon 4 for each sample analyzed.
9. **Save the Run Setup and then transfer the file to a USB memory stick.**
10. **Print a list of required volumes of reagents and the plate setup. Select "Pre Run Information" from the "Tools" menu and print the resulting report.**
11. **Proceed with the protocol "Immobilization of PCR Products to Streptavidin Sepharose HP Beads for Analysis Using the PyroMark Q24", page 18.**

    Note: For reliable results, we recommend single peak heights above 30 relative light units (RLU).

    Note: 30 RLU can be set as the required peak height for passed quality in assay setup (see the *PyroMark Q24 User Manual*)

Protocol: Immobilization of PCR Products to Streptavidin Sepharose HP Beads for Analysis Using the PyroMark Q24

This protocol is for immobilization of template DNA to Streptavidin Sepharose HP beads for subsequent analysis on the PyroMark Q24.

Things to do before starting

- Allow all required reagents and solutions to reach room temperature before starting.

Procedure

1. **Gently shake the bottle containing Streptavidin Sepharose HP beads until a homogenous solution is obtained.**
2. **For each sample, prepare a solution for DNA immobilization as described in Table 4.**

   Note: Prepare a master mix with the components listed in Table 4. Aliquot the master mix to a PCR plate or strips and then add the required volume of PCR product. Adjust the volume of RNase-free water according to the volume of PCR product. Prepare a volume 10% greater than the number of samples to be analyzed.

   Note: The total volume per well should be 80 µl after addition of the master mix and PCR product.

Table 4. DNA immobilization components

	Volume per sample
Master mix component:	
Streptavidin Sepharose HP beads	2 µl
PyroMark Binding Buffer	40 µl
RNase-free water	28–33 µl
PCR product	**5–10 µl**
Total volume	**80 µl**

3. **Seal the PCR plate (or strips) using strip caps.**

   Note: Ensure that no leakage is possible between the wells.

4. **Agitate the PCR plate (or strips) constantly for 5–10 minutes at room temperature (15–25°C) using a mixer (1400 rpm).**

   During immobilization, prepare the PyroMark Q24 Vacuum Workstation for sample preparation (see Appendix B, page 50).

5. **Proceed immediately with protocol "Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q24", page 20.**

   Note: Sepharose beads sediment quickly and capturing of beads must take place immediately once the agitation is complete.

Protocol: Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q24

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

Important point before starting

- PyroMark Denaturation Solution contains sodium hydroxide, which is irritating to eyes and skin. Be sure to following the safety instructions included with the reagent bottle.

Things to do before starting

- Before opening the tubes containing sequencing primers, spin briefly to collect contents at the bottom of the tubes.
- Dissolve each sequencing primer in 180 µl high-purity water (Milli-Q 18.2MΩ x cm or equivalent, filtered through 0.22 µm filter) to a final concentration of 10 µM.
- Dilute each sequencing primer to 0.3 µM in Annealing Buffer.
- Carefully plan the addition of sequencing primers to the PyroMark Q24 Plate. Sequencing primers must be added in the same pattern as predefined in the plate setup, depending on which region (exon 2 or exon 4) is to be analyzed. Pyrosequencing reactions of the two different regions are performed in separate wells.
- Prepare the vacuum workstation as described in Appendix A, page 50.
- Pre-warm a PyroMark Q24 Plate Holder to 80°C.

Procedure

1. **Add 25 µl diluted sequencing primer (0.3 µM) to the wells to be analyzed on the PyroMark Q24 Plate.**

   Sequencing primers for exon 2 and exon 4 are added to separate wells, as predefined in the plate setup.

   Use one of the supplied PyroMark Q24 Plate Holders for support when preparing and moving the plate.

2. **Place the PCR plate (or strips) and PyroMark Q24 Plate in the workstation.**

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

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

4. **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 s. Take care when picking up the tool.**

   Note: Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.

5. **Transfer the tool to the trough containing 70 % ethanol (trough 1). Flush the filter probes for 5 s.**

PyroMark Q24 Vacuum Workstation.

6. **Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
7. **Transfer the tool to the trough containing Wash Buffer (trough 3). Flush the filter probes for 10 s.**
8. **Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.**

PyroMark Q24 vacuum tool.

9. **While holding the tool over the PyroMark Q24 Plate, turn the vacuum switch off.**
10. **Release the beads into the plate containing 0.3 µM sequencing primer in 25 µl Annealing Buffer, by gently shaking the tool in the wells.**
11. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
12. **Wash the filter probes by lowering the probes into high purity water (trough 5) and applying vacuum. Flush the probes with 70 ml high-purity water.**
13. **Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.**
14. **Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.**
15. **Turn off the vacuum pump.**

    Note: At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage, see Appendix B, page 50.
16. **Heat the PyroMark Q24 Plate with the samples at 80°C for 2 min using the pre-warmed PyroMark Q24 Plate Holder.**

    Note: The Plate Holder is supplied with the vacuum workstation.
17. **Allow the samples to cool to room temperature (15–25°C) for at least 5 min.**
18. **Proceed with the protocol "Genotyping of the HFE Gene Using the PyroMark Q24", page 23.**

Protocol: Genotyping of the HFE Gene Using the PyroMark Q24

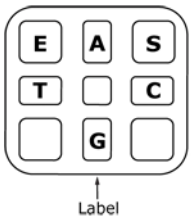
This protocol describes loading of PyroMark Gold Q24 Reagents into the PyroMark Q24 Cartridge and analysis of SNPs in exons 2 and 4 of the HFE gene using the PyroMark Q24. For a detailed description of how to set up a run, see the *PyroMark Q24 User Manual*, "Setting up a run".

Important points before starting

- Switch on the instrument (see the *PyroMark Q24 User Manual*).
- The Pre Run Information report provides information about the volume of nucleotides, enzyme, and substrate mixtures needed for a specific run.
- Allow the enzyme and substrate mixtures and cartridge to reach room temperature (15–25°C).

Procedure

1. **Place the reagent cartridge with the label facing toward you.**



PyroMark Q24 Cartridge, as seen from above. **E**: Enzyme Mixture; **S**: Substrate Mixture; **G**: dGTP; **C**: dCTP; **T**: dTTP; **A**: dATPαS.

2. **Load the cartridge with the appropriate volumes of nucleotides, enzyme, and substrate mixtures according to the volume information given in the Pre Run Information of the PyroMark Q24 Software. Ensure that no air bubbles are transferred from the pipet to the cartridge.**
3. **Open the instrument lid and insert the reagent filled cartridge as described in the *PyroMark Q24 User Manual*.**
4. **Open the plate-holding frame and place the plate on the heating block. Close the plate-holding frame and the instrument lid.**
5. **Insert the USB stick (containing the run file) into the USB port at the front of the instrument.**

   Note: Do not remove the USB stick before the run is finished.
6. **Select "Run" in the main menu (using the ▼ and ▲ screen buttons) and press "OK".**

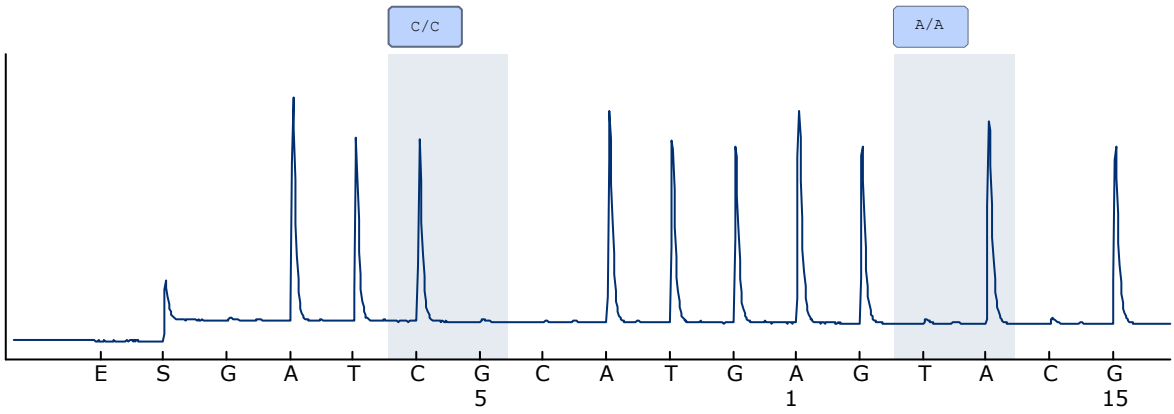
7. **Select the run file using the ▼ and ▲ screen buttons.**

   Note: To view the contents of a folder, select the folder and press "Select". To go back to the previous view, press "Back".

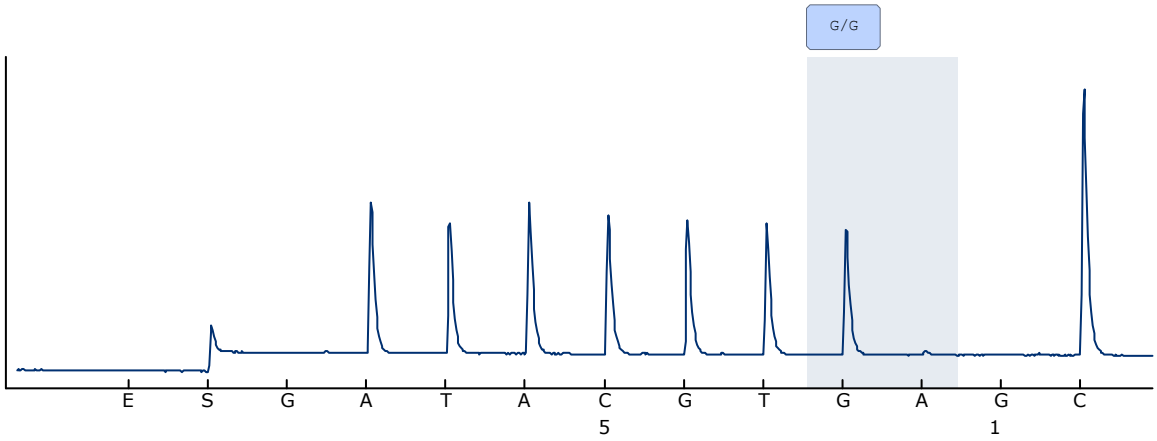
8. **When the run file is selected, press "Select" to start the run.**
9. **When the run is finished and the instrument confirms that the run file has been saved to the memory stick, press "Close".**
10. **Remove the USB stick.**
11. **Open the instrument lid.**
12. **Open the cartridge gate and remove the cartridge.**
13. **Close the cartridge gate.**
14. **Open the plate-holding frame and remove the plate from the heating block.**
15. **Close the plate-holding frame and the instrument lid.**
16. **Discard the plate and wash the cartridge (see the *PyroMark Q24 User Manual*).**
17. **Open the run file on the USB memory stick and analyze the data in AQ mode of the PyroMark Q24 Software. For details about how to analyze a run, see the *PyroMark Q24 Software Manual*.**
18. **To view the genotyping result and quality assessment go to "Menu" and "SNP Overview Report". Pyrograms are shown in the "SNP Full Report" or "SNP Pyrogram Report". See below for examples of resulting Pyrogram traces.**

    Note: For reliable results, we recommend single peak heights above 30 relative light units (RLU).

    Note: 30 RLU can be set as the required peak height for passed quality in assay setup (see the *PyroMark Q24 User Manual*)



Pyrogram trace obtained after analysis of samples with genotypes C/C in H63D and A/A in S65C.



Pyrogram trace obtained after analysis of samples with genotype G/G in C282Y.

Protocol: Assay and Run Setup Using PyroMark Q96 ID Software

This protocol is for creating an Entry Setup to assign assay parameters, and a Run Setup for analysis of SNPs H63D and S65C in exon 2 and SNP C282Y in exon 4 of the HFE gene using the PyroMark Q96 ID.

The two exons each require a different Assay Setup. This protocol includes instructions for setting up both assays.

Important points before starting

- For further information on how to create a Run Setup, see the *PyroMark Q96 ID Software Online Help*.
- Assays for exons 2 and 4 are performed in separate wells, but can be run on the same plate.
- Steps 1–4 are only performed the first time the assay is run.

Protocol

1. **Set up a simplex entry for analysis of SNPs in exon 2 of the HFE gene. Use the following sequence and dispensation order.**

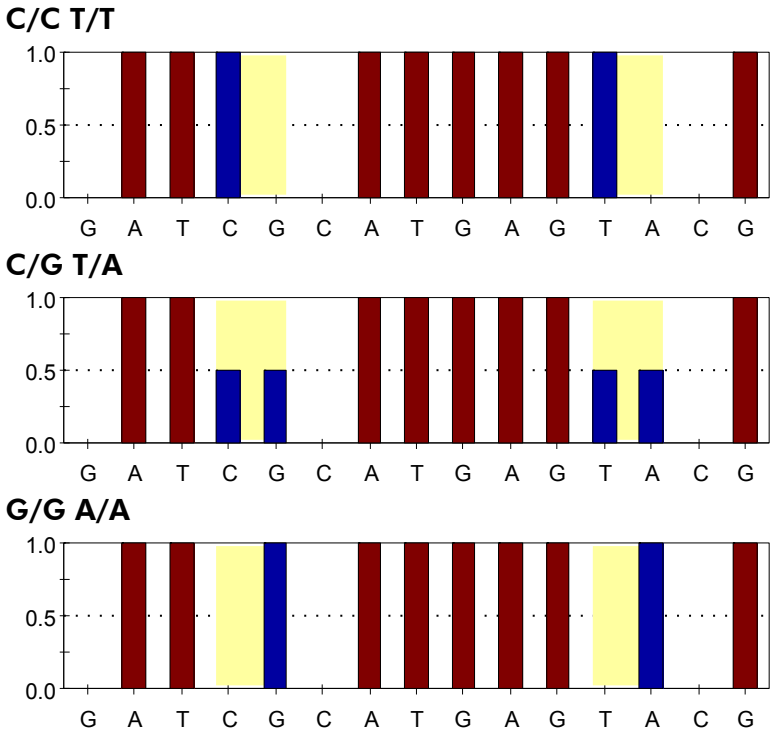
   Sequence to analyze:

   ATC/GATGAGT/AGT

   Manually enter the following dispensation order:

   GATCGCATGAGTACG

2. **Save the Entry as "HFE_exon2".**



Selected theoretical outcome for H63D and S65C in exon 2 of the HFE gene using PyroMark Q96 ID Software.

3. **Set up a simplex entry for analysis of SNP in exon 4 of the HFE gene. Use the following sequence and dispensation order.**

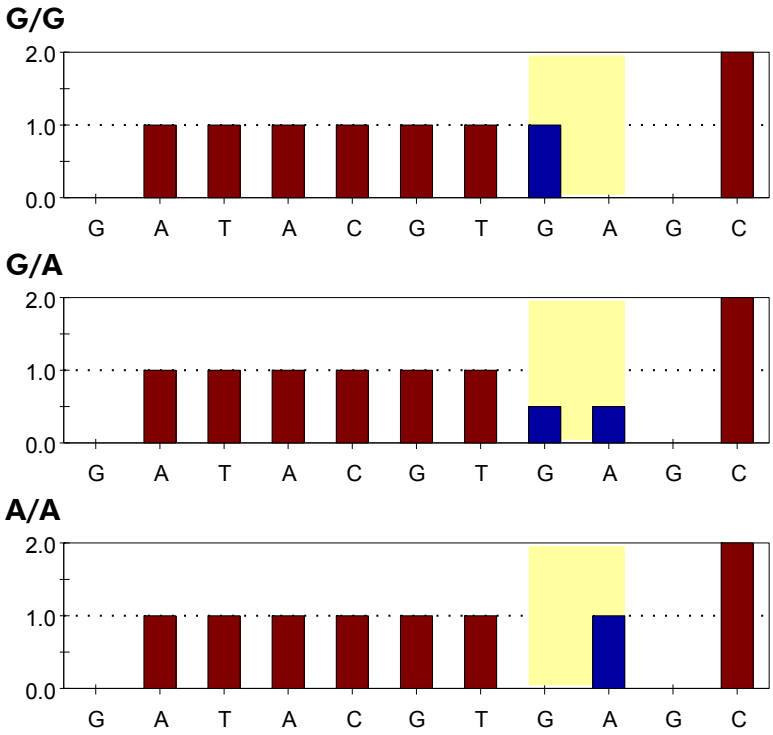
   Sequence to analyze:

   ATACGTG/ACCA

   Manually enter the following dispensation order:

   GATACGTGAGC

4. **Save the Entry as "HFE_exon4".**



Theoretical outcomes for C282Y in exon 4 of the HFE gene using PyroMark Q96 ID Software.

5. **Select "SNP → SNP Runs" in the main menu.**
   The tree view for this sub-module opens in the tree view area.
6. **Right-click on a folder in the tree view and select "New SNP Run". The "SNP Run Setup" dialog opens.**
   The Run Setup will be saved in the selected folder.
7. **Design the plate layout by selecting instrument parameters and by adding the assay setup (HFE_exon2 or HFE_exon4) to the same number of wells as samples to analyze.**
8. **Click "Save" to save the Run setup.**
9. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup.**
   Note: Click "View" and choose "Run" in the web browser area.
10. **Proceed with protocol "Immobilization of PCR Products to Streptavidin Sepharose HB Beads for Analysis on the PyroMark Q96 ID", page 29.**

Protocol: Immobilization of PCR Products to Streptavidin Sepharose HP Beads for Analysis Using the PyroMark Q96 ID

This protocol is for immobilization of template DNA to Streptavidin Sepharose HP beads for subsequent analysis on the PyroMark Q96 ID.

Things to do before starting

- Allow all required reagents and solutions to reach room temperature before starting.

Procedure

1. **Gently shake the bottle containing Streptavidin Sepharose HP beads until a homogenous solution is obtained.**
2. **For each sample, prepare a solution for DNA immobilization as described in Table 5.**

   Note: Prepare a master mix with the components listed in Table 5. Aliquot the master mix to a PCR plate or strips and then add the required volume of PCR product. Adjust the volume of RNase-free water according to the volume of PCR product. Prepare a volume 10% greater than the number of samples to be analyzed.

   Note: The total volume per well should be 80 µl after addition of the master mix and PCR product.

   Note: Assays for exons 2 and 4 are performed in separate wells, but can be run on the same plate.

Table 5. DNA immobilization components

	Volume per sample
Master mix component:	
Streptavidin Sepharose HP beads	3 µl
PyroMark Binding Buffer	40 µl
RNase-free water	27 µl
PCR product	**10 µl**
Total volume	**80 µl**

3. **Seal the PCR plate (or strips) using strip caps.**

   Note: Ensure that no leakage is possible between the wells.

4. **Agitate the PCR plate (or strips) constantly for 5–10 minutes at room temperature (15–25°C) using a mixer (1,400 rpm).**

   During immobilization, prepare the PyroMark Q96 Vacuum Workstation for sample preparation (see Appendix B, page 50).

5. **Proceed immediately with protocol "Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q96 ID", page 31.**

   Note: Sepharose beads sediment quickly and capturing of beads must take place immediately once the agitation is complete.

Protocol: Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q96 ID

This protocol is for the preparation of single-stranded DNA and annealing of the sequencing primer to the template before Pyrosequencing analysis using the PyroMark Q96 ID.

Important point before starting

- PyroMark Denaturation Solution contains sodium hydroxide, which is irritating to eyes and skin. Be sure to following the safety instructions included with the reagent bottle.

Things to do before starting

- Before opening the tubes containing sequencing primers, spin briefly to collect contents at the bottom of the tubes.
- Dissolve each sequencing primer in 180 µl high-purity water (Milli-Q 18.2MΩ x cm or equivalent, filtered through 0.22 µm filter) to a final concentration of 10 µM.
- Dilute each sequencing primer to 0.4 µM in Annealing Buffer.
- Carefully plan the addition of the sequencing primer to the PyroMark Q96 Plate Low. The sequencing primers must be added to the same wells as predefined in the plate setup.
- Prepare the vacuum workstation as described in Appendix B, page 50.
- Pre-warm a PyroMark Q96 Sample Prep Thermoplate Low to 80°C.

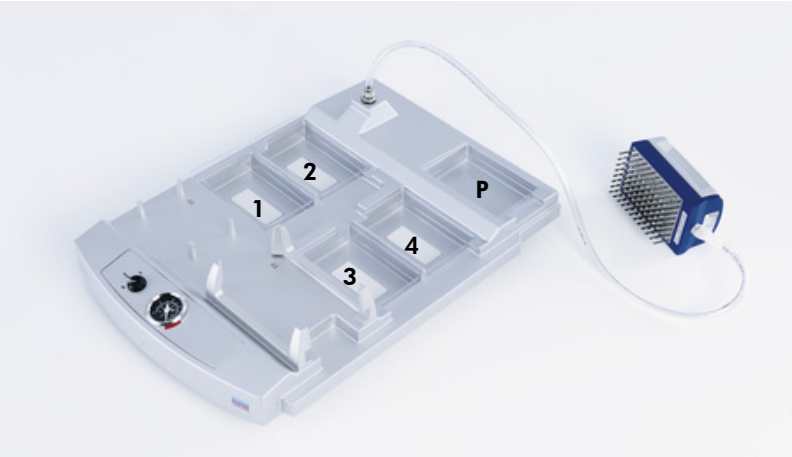
Procedure

1. **Add 40 µl diluted sequencing primer (0.4 µM) to the wells to be analyzed of a PyroMark Q96 Plate Low, according to the plate set up in the protocol "Entry and Run Setup Using PyroMark Q96 ID Software".**
2. **Place the PCR plate (or strips) and PyroMark Q96 Plate Low on the worktable of PyroMark Q96 Vacuum Workstation.**

   Ensure that the plate is in the same orientation as when samples were loaded.
3. **Apply vacuum to the tool by opening the vacuum switch on the workstation.**

4. **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 s. Pick up the tool carefully.**

   Note: Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.



Placement of PCR plate (or strips) and PyroMark Q96 Plate Low (PM Plate) on the PyroMark Q96 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4** and **P**).

5. **Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.**
6. **Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
7. **Transfer the tool to the trough containing Wash Buffer (trough 3). Flush the filter probes for 10 s.**
8. **Raise the tool to beyond 90° vertical for 5 s to drain liquid from the filter probes.**

PyroMark Q96 vacuum tool.

9. **While holding the tool over the PyroMark Q96 Plate Low, close the vacuum switch on the workstation.**
10. **Release the beads into the wells containing sequencing primer by gently shaking the tool in the wells.**
11. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
12. **Wash the filter probes by lowering the probes into high-purity water (parking position) and applying vacuum. Flush the probes with 70 ml high-purity water.**
13. **Raise the tool to beyond 90° vertical for 5 s to drain liquid from the filter probes.**
14. **Close the vacuum switch on the workstation, and place the tool in the "Parking" position.**
15. **Turn off the vacuum pump.**

    Note: At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q96 Vacuum Workstation should be checked for dust and spillage, see Appendix A, page 50.
16. **Heat the PyroMark Q96 Plate Low with the samples at 80°C for 2 min using a prewarmed PyroMark Q96 Sample Prep Thermoplate Low and a heating block.**
17. **Remove the PyroMark Q96 Plate from the plate holder, and let the samples cool to room temperature (15–25°C) for at least 5 min.**
18. **Proceed with the protocol "Genotyping of Exons 2 or 4 of HFE Using the PyroMark Q96 ID ", page 34.**

Protocol: Genotyping of Exons 2 or 4 of HFE Using the PyroMark Q96 ID

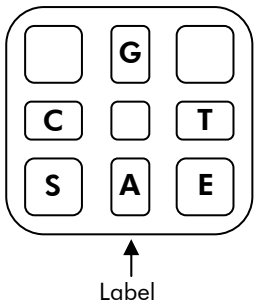
This protocol describes loading of PyroMark Gold Q96 Reagents into the PyroMark Q96 Cartridge and genotyping analysis of the HFE gene using the PyroMark Q96 ID. For a detailed description about how to set up a run, see *PyroMark Q96 ID Software Online Help*.

Things to do before starting

- Switch on the instrument (see the *PyroMark Q96 ID User Manual*).
- Allow all reagents and solutions to reach room temperature (15–25°C) before starting.
- PyroMark Q96 ID Software provides the volume of nucleotides, enzyme mixture, and substrate mixture needed for a specific run. In the Browser area of the PyroMark Q96 ID Software click "View" and choose "Run" to see these volumes.

Protocol

1. **Load the PyroMark Q96 Cartridge with the appropriate volumes of PyroMark Gold Q96 Reagents as shown below.**

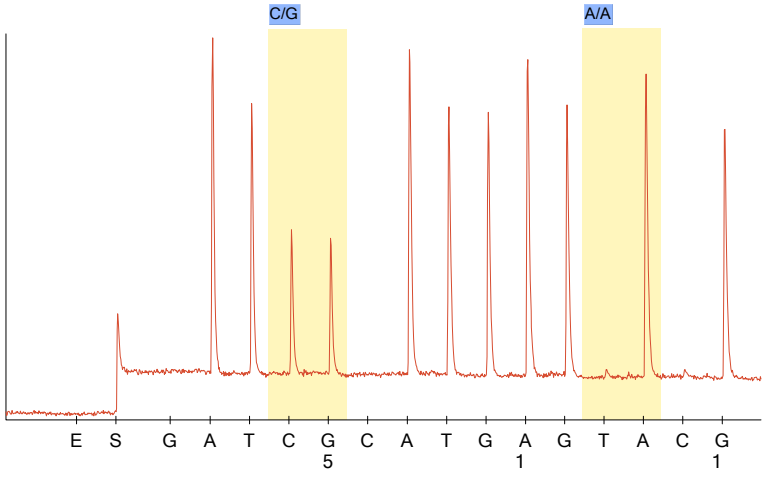


Schematic diagram of the PyroMark Q96 Cartridge (viewed from above). E: Enzyme Mixture; **S**: Substrate Mixture; **G**: dGTP; **C**: dCTP; **T**: dTTP; **A**: dATPαS.

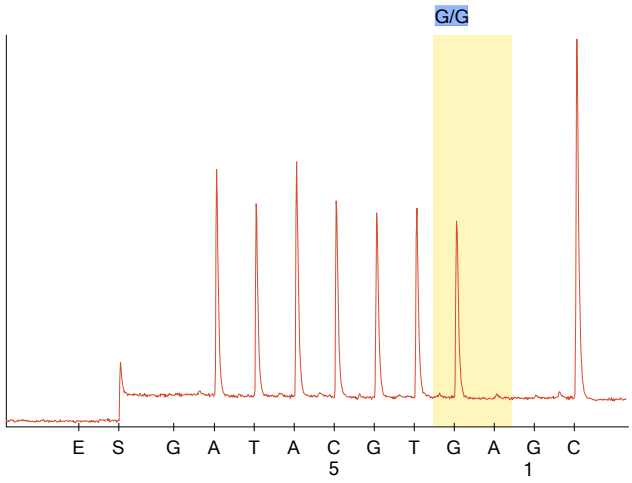
2. **Open the instrument lid and the process chamber lid.**
3. **Open the plate-holding frame and place the PyroMark Q96 Plate Low on the heating block.**
4. **Close the plate-holding frame and the processing chamber lid.**
5. **Open the dispensing unit cover, release the latch, and then open the cover.**
6. **Insert the filled reagent cartridge with the label facing you.**
7. **Close the dispensing unit cover. Ensure that the latch snaps into its locked position.**

8. **Close the instrument lid and perform the run (see the *PyroMark Q96 ID User Manual*).**
9. **When the run is complete, open the instrument lid.**
10. **Open the dispensing unit and remove the reagent cartridge by lifting it up and pulling it out.**
11. **Close the dispensing unit.**
12. **Open the process chamber lid and remove the PyroMark Q96 Plate Low from the heating block (see the *PyroMark Q96 ID User Manual*).**
13. **Close the process chamber and the instrument lid (see the *PyroMark Q96 ID User Manual*).**
14. **Discard the PyroMark Q96 Plate Low and clean the PyroMark Q96 Cartridge (see the *PyroMark Gold Q96 Reagents Handbook*).**
15. **Open the run in the PyroMark Q96 ID Software and analyze all wells (see *PyroMark Q96 ID Software Online Help*). The analysis results (genotypes) and quality assessment are displayed above the variable position in the Pyrogram® trace.**

    Note: For reliable results, we recommend single peak heights above 15 RLU. The mean single peak height for a well should be at least 15 RLU.



Pyrogram trace obtained after analysis of samples with genotypes C/G in H63D and A/A in S65C.



Pyrogram trace obtained after analysis of samples with genotype G/G in C282Y.

Protocol: Assay and Run Setup Using PyroMark Q96 MD Software

This protocol is for creating an Entry Setup to assign assay parameters, and a Run Setup for analysis of SNPs H63D and S65C in exon 2 and SNP C282Y in exon 4 of the HFE gene using the PyroMark Q96 MD.

The two exons each require a different Assay Setup. This protocol includes instructions for setting up the two Assays.

Important points before starting

- For further information on how to create a Run Setup, see the *PyroMark Q96 MD Software Online Help*.
- Assays for exons 2 and 4 are performed in separate wells, but can be run on the same plate.
- Steps 1–4 are only performed the first time the assay is run.

Protocol

1. **Set up a simplex entry for analysis of SNPs in exon 2 of the HFE gene. Use the following sequence and dispensation order.**

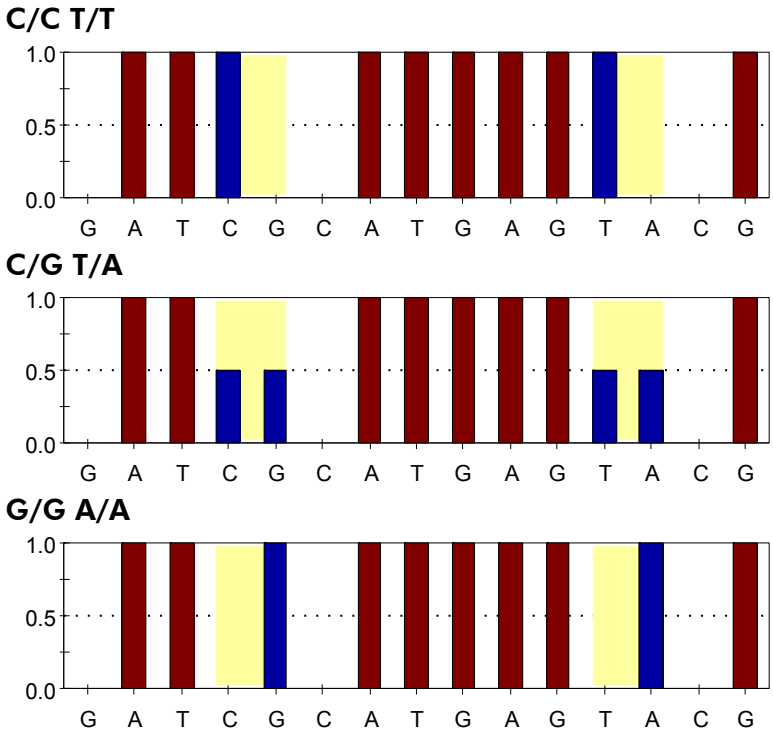
   Sequence to analyze:

   ATC/GATGAGT/AGT

   Manually enter the following dispensation order:

   GATCGCATGAGTACG

2. **Save the Entry as "HFE_exon2".**



Selected theoretical outcome for H63D and S65C in exon 2 of the HFE gene using PyroMark Q96 MD Software.

3. **Set up a simplex entry for analysis of SNP in exon 4 of the HFE gene. Use the following sequence and dispensation order.**

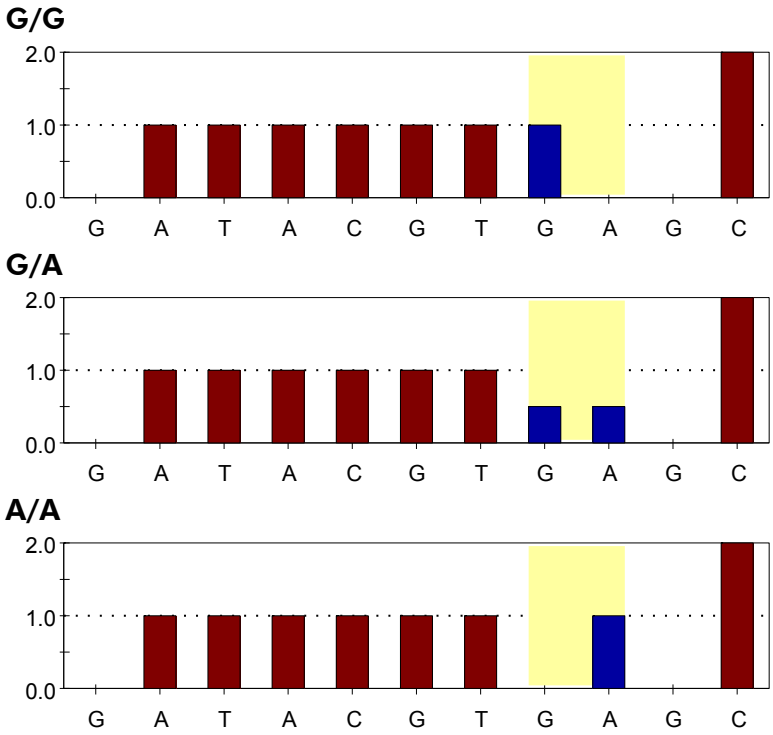
   Sequence to analyze:

   ATACGTG/ACCA

   Manually enter the following dispensation order:

   GATACGTGAGC

4. **Save the Entry as "HFE_exon4".**



Theoretical outcomes for C282Y in exon 4 of the HFE gene using PyroMark Q96 MD Software.

5. **Select "SNP → SNP Runs" in the main menu.**
   The tree view for this sub-module opens in the tree view area.
6. **Right-click on a folder in the tree view and select "New SNP Run". The "SNP Run Setup" dialog opens.**
   The Run Setup will be saved in the selected folder.
7. **Design the plate layout by selecting instrument parameters and by adding the assay setup (HFE_exon2 or HFE_exon4) to the same number of wells as samples to analyze.**
8. **Click "Save" to save the Run setup.**
9. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup.**
   Note: Click "View" and choose "Run" in the web browser area.
10. **Proceed with protocol "Immobilization of PCR Products to Streptavidin Sepharose HB Beads for Analysis on the PyroMark Q96 MD", page 29.**

Protocol: Immobilization of PCR Products to Streptavidin Sepharose HP Beads for Analysis Using the PyroMark Q96 MD

This protocol is for immobilization of template DNA to Streptavidin Sepharose HP beads for subsequent analysis on the PyroMark Q96 MD.

Things to do before starting

- Allow all required reagents and solutions to reach room temperature before starting.

Procedure

1. **Gently shake the bottle containing Streptavidin Sepharose HP beads until a homogenous solution is obtained.**
2. **For each sample, prepare a solution for DNA immobilization as described in Table 6.**

   Note: Prepare a master mix with the components listed in Table 6. Aliquot the master mix to a PCR plate or strips and then add the required volume of PCR product. Adjust the volume of RNase-free water according to the volume of PCR product. Prepare a volume 10% greater than the number of samples to be analyzed.

   Note: The total volume per well should be 80 µl after addition of the master mix and PCR product.

   Note: Assays for exons 2 and 4 are performed in separate wells, but can be run on the same plate.

Table 6. DNA immobilization components

	Volume per sample
Master mix component:	
Streptavidin Sepharose HP beads	2 µl
PyroMark Binding Buffer	40 µl
RNase-free water	28–33 µl
PCR product	**5–10 µl**
Total volume	**80 µl**

3. **Seal the PCR plate (or strips) using strip caps.**

   Note: Ensure that no leakage is possible between the wells.

4. **Agitate the PCR plate (or strips) constantly for 5–10 minutes at room temperature (15–25°C) using a mixer (1,400 rpm).**

   During immobilization, prepare the PyroMark Q96 Vacuum Workstation for sample preparation (see Appendix A, page 50).

5. **Proceed immediately with protocol "Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q96 MD", page 42.**

   Note: Sepharose beads sediment quickly and capturing of beads must take place immediately once the agitation is complete.

Protocol: Preparation of Samples for Pyrosequencing Analysis Using the PyroMark Q96 MD

This protocol is for the preparation of single-stranded DNA and annealing of the sequencing primer to the template before Pyrosequencing analysis using the PyroMark Q96 MD.

Important points before starting

- PyroMark Denaturation Solution contains sodium hydroxide, which is irritating to eyes and skin. Be sure to following the safety instructions included with the reagent bottle.

Things to do before starting

- Before opening the tubes containing sequencing primers, spin briefly to collect contents at the bottom of the tubes.
- Dissolve the sequencing primer in 180 µl high-purity water (Milli-Q 18.2MΩ x cm or equivalent, filtered through 0.22 µm filter) to a final concentration of 10 µM.
- Dilute each sequencing primer to 0.3 µM in Annealing Buffer.
- Carefully plan the addition of sequencing primers to the PyroMark Q96 HS Plate. The sequencing primers must be added to the same wells as predefined in the plate setup, depending on which region (exon 2 or exon 4) is to be analyzed. Pyrosequencing reactions of the two different regions are performed in separate wells.
- Prepare the vacuum workstation as described in Appendix A, page 50.
- Pre-warm a PyroMark Q96 HS Sample Prep Thermo Plate to 80°C.

Procedure

1. **Add 12 µl diluted sequencing primer (0.3 µM) to the wells to be analyzed of a PyroMark Q96 HS Plate, according to the plate set up in "Assay and Run set up using PyroMark Q96 MD Software".**
2. **Place the PCR plate (or strips) and the PyroMark Q96 HS Plate on the worktable of the PyroMark Q96 Vacuum Workstation.**

   Ensure that the plate is in the same orientation as when samples were loaded.
3. **Apply vacuum to the tool by opening the vacuum switch on the workstation.**

4. **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 s. Pick up the tool carefully.**

   Note: Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.

Placement of PCR plate (or strips) and PyroMark Q96 HS Plate (PM Plate) on the PyroMark Q96 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4** and **P**).

5. **Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.**
6. **Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
7. **Transfer the tool to the trough containing Wash Buffer (trough 3). Flush the filter probes for 10 s.**
8. **Raise the tool beyond 90° vertical, for 5 s to drain liquid from the filter probes.**

PyroMark Q96 vacuum tool.

9. **While holding the vacuum tool over the PyroMark Q96 HS Plate, close the vacuum switch on the workstation.**
10. **Release the beads into the wells containing sequencing primer by gently shaking the tool in the wells.**
11. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
12. **Wash the filter probes by lowering the probes into high-purity water (parking position) and applying vacuum. Flush the probes with 70 ml high-purity water.**
13. **Raise the tool beyond 90° vertical, for 5 s to drain liquid from the filter probes.**
14. **Close the vacuum switch on the workstation, and place the tool in the Parking (P) position.**
15. **Turn off the vacuum pump.**

    Note: At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q96 Vacuum Workstation should be checked for dust and spillage, see Appendix A, page 50.
16. **Heat the PyroMark Q96 HS Plate with the samples at 80°C for 2 min using a heating block and the prewarmed PyroMark HS Q96 Sample Prep Thermo Plate.**

    Note: Use one Sample Prep Thermo Plate as lid on the plate to prevent evaporation of the samples.
17. **Remove the PyroMark Q96 HS Plate from the thermo plate, and allow the samples cool to room temperature (15–25°C) for at least 5 min.**
18. **Proceed with the protocol "Genotyping of Exons 2 and 4 of HFE Using the PyroMark Q96 MD", page 45.**

Protocol: Genotyping of Exons 2 and 4 of HFE Using the PyroMark Q96 MD

This protocol describes loading of PyroMark Gold Q96 Reagents into the PyroMark Q96 HS Reagent Tips (RDTs) and Capillary Tips (CDTs) and SNP analysis of the HFE gene using the PyroMark Q96 MD. If using the PyroMark Q96 HS Nucleotide Tips and PyroMark Q96 Dispensing Tip Holder, please refer to the *PyroMark Q96 HS Nucleotide Tip Product Sheet* for filling instructions. For a detailed description about how to set up a run, see *PyroMark Q96 MD Online Help*.

Things to do before starting

- Switch on the instrument (see the *PyroMark Q96 MD User Manual*).
- Allow all reagents and solutions to reach room temperature (15–25°C) before starting.
- PyroMark Q96 MD Software provides the volume of nucleotides, enzyme mixture, and substrate mixture needed for a specific run. In the Browser area of the PyroMark Q96 ID Software, click "View" and choose "Run" to see these volumes.

Protocol

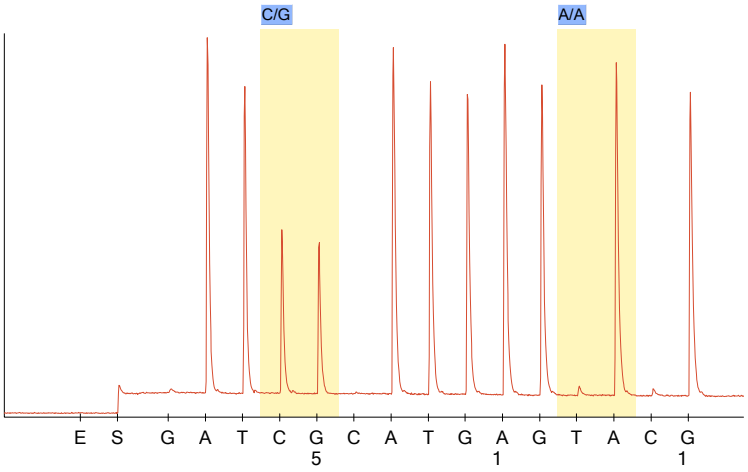
1. **Load the PyroMark Q96 Reagent Tips and Capillary Tips in the PyroMark Q96 Dispensing Tip Holder with the appropriate volumes of PyroMark Gold Q96 Reagents.**

Arrangement of tips in the PyroMark Q96 Capillary Tip Holder.
E: Enzyme Mixture; **S**: Substrate Mixture; **G**: dGTP; **C**: dCTP; **T**: dTTP; **A**: dATPαS.

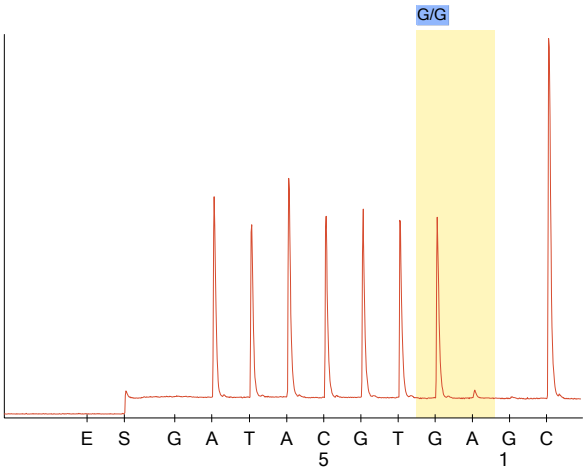
2. **Open the processing chamber lid using the software.**
3. **Place the PyroMark Q96 HS Plate on the heating block. Close the process chamber lid.**

4. **Open the dispensing unit cover by releasing the latch. With the two RDTs furthest away from you, insert the filled dispensing tip holder into position.**
5. **Close the dispensing unit cover. Ensure that the latch snaps into its locked position.**
6. **Close the instrument lid and perform the run (see the *PyroMark Q96 MD User Manual*).**
7. **After the run has finished, open the instrument lid.**
8. **Open the dispensing unit and remove the dispensing tip holder and the PyroMark Q96 HS plate.**
9. **Close the dispensing unit and the instrument lid (see the *PyroMark Q96 MD User Manual*).**
10. **Discard the PyroMark Q96 HS Plate and clean the tips in the PyroMark Q96 HS Dispensing tip holder (see the *PyroMark Gold Q96 Reagents Handbook*).**
11. **Open the run in the PyroMark Q96 MD Software and analyze all wells (see *PyroMark Q96 Software Online Help* for more information). The analysis results (genotypes) and quality assessment are displayed above the variable position in the Pyrogram trace.**

    Note: For reliable results, we recommend single peak heights above 100 RLU. The mean single peak height for a well should be at least 100 RLU.



Pyrogram trace obtained after analysis of samples with genotypes C/G in H63D and A/A in S65C.



Pyrogram trace obtained after analysis of samples with genotype G/G in C282Y.

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).

	Comments and suggestions
Low or missing peaks in the Pyrogram	
a) PCR failed	Check the PCR samples using a gel technique to confirm that there is one strong, specific band. If not, rerun the PCR using high-quality DNA.
b) The wells marked in the run setup do not agree with the sample placement on the plate for immobilization	Check that the PCR plate (or strips) was loaded on the vacuum workstation according to the plate setup.
c) One or several of the reagent compartments in the dispensing unit were not correctly filled	Be sure to add sufficient reagents in the correct compartment of the dispensing unit.
d) One of the reagent needles in the dispensing unit is blocked or damaged	Clean the dispensing unit and check that it is working correctly. For detailed instructions, see the user manual of your PyroMark instrument. In case of bent needles, discard the dispensing unit according to federal, state, and local environmental regulations for disposal of laboratory waste.
e) The reagent cartridge or tip holder is inserted incorrectly	Ensure that the reagent cartridge or dispensing tip holder is inserted correctly.

	Comments and suggestions
f) Low signal due to dirty light guides or lens array	**PyroMark Q24**: Clean the heating block and light guides; see section 6.2.2 of the *PyroMark Q24 User Manual*. **PyroMark Q96 ID**: Clean the heating block and lens array; see sections 7.3 and 7.4 of the *PyroMark Q96 ID User Manual*. **PyroMark Q96 MD**: Clean the heating block and lens array; see sections 7.4 and 7.5 of the *PyroMark Q96 MD User Manual*.
g) Filter probes not working correctly	**PyroMark Q24**: Test the filter probes and ensure they are working correctly. See section 6.3.2 of the *PyroMark Q24 User Manual*. **PyroMark Q96 ID**: Test the filter probes and ensure they are working correctly. See section 7.8 of the *PyroMark Q96 ID User Manual*. **PyroMark Q96 MD**: Test the filter probes and ensure they are working correctly. See section 7.12 of the *PyroMark Q96 MD User Manual*.
Poor or faulty sequence	
a) Incorrect sequence to analyze	Check typing and reference sequence.
b) Nucleotides incorrectly diluted or stored	Be sure to follow the instructions in the *PyroMark Gold Q24 Reagents Handbook* or *PyroMark Gold Q96 Reagents Handbook*.
c) Crosstalk (light from one well appears in the neighboring well)	Avoid placing assays with high signals close to assays with low signals.
d) Dispensation error	Replace the reagent cartridge. If the problem remains, contact QIAGEN Technical Service.

Appendix A: Preparation of the PyroMark Q24 Vacuum Workstation or PyroMark Q96 Vacuum Workstation

This protocol describes how to prepare the PyroMark Q24 Vacuum Workstation or PyroMark Q96 Vacuum Workstation before preparation of single-stranded DNA.

Important point before starting

- PyroMark Denaturation Solution contains sodium hydroxide, which is irritating to eyes and skin. Be sure to following the safety instructions included with the reagent bottle.

Procedure

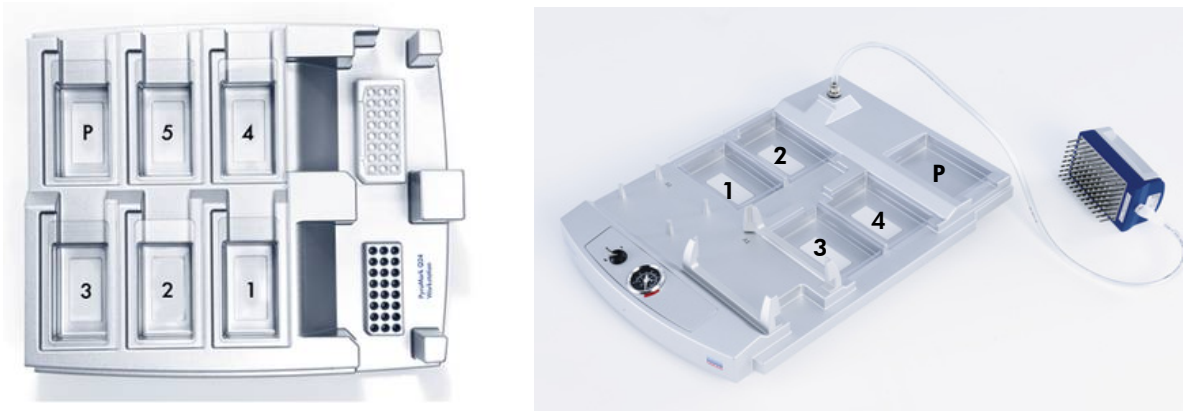
1. **Fill five separate troughs (supplied with the PyroMark Q24 or PyroMark Q96 Vacuum Workstations) according to Table 7.**

   A suggested setup is shown on the next page. Refill the troughs to these levels whenever necessary.

Table 7. Vacuum workstation volumes

Trough	Solution	PyroMark Q24 Vacuum Workstation	PyroMark Q96 Vacuum Workstation
1	Ethanol (70%)	50 ml	110 ml
2	Denaturation Solution	40 ml	90 ml
3	Wash Buffer	50 ml	110 ml
4	High-purity water	50 ml	110 ml
5	High-purity water	70 ml	–
P	High-purity water	–	180 ml

A B



A PyroMark Q24 Vacuum Workstation. B PyroMark Q96 Vacuum Workstation.

2. **Switch on the vacuum pump.**
3. **Apply vacuum to the tool by opening the vacuum switch.**
4. **PyroMark Q24 Vacuum Workstation: Wash the filter probes by lowering the probes into trough 5 and flushing them with 70 ml high-purity water.**

   PyroMark Q96 Vacuum Workstation: Wash the filter probes by lowering the probes into the Parking Position (trough P) and flushing them with 180 ml high-purity water.

   Ensure that the water is being transferred to the waste container. If not, ensure that the tubing is connected correctly and is not broken. Broken tubing should be replaced, see the *PyroMark Q24 User Manual* or *PyroMark Q96 User Manual* section on replacing the tubing.
5. **PyroMark Q24 Vacuum Workstation only: Ensure that the waste filter is dry. If the filter is wet, it should be replaced, see the *PyroMark Q24 User Manual* section on replacing the tubing.**
6. **Refill trough 5 with 70 ml high-purity water or Parking Position with 180 ml high-purity water.**
7. **Close the vacuum switch and place the tool in the Parking (P) position.**

Appendix B: Emptying the Waste Container and Troughs

WARNING	**Hazardous chemicals**
	The Denaturation Solution used with the PyroMark Q24 Vacuum Workstation or PyroMark Q96 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 Material Safety Data Sheets (MSDSs) 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.

The following item is required:

- High-purity water (Milli-Q 18.2 MΩ x cm, www.millipore.com, or equivalent).

Procedure

1. **Ensure that no vacuum is applied to the vacuum tool, the vacuum switch is closed (Off), and the vacuum pump is switched off.**
2. **Discard any solutions left in the troughs.**
3. **Rinse the troughs with high-purity water, or replace them, if necessary.**
4. **Empty the waste container.**

   The cap can be removed without disconnecting the tubing.
5. **If the PyroMark Q24 Vacuum Workstation or PyroMark Q96 Vacuum Workstation must be cleaned (for dust or spillage), follow the instructions in relevant manual (*PyroMark Q24 User Manual*, the *PyroMark Q96 ID User Manual* or *PyroMark Q96 MD User Manual*).**

References

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Product	Contents	Cat. no.
PyroMark HFE	Genotyping assay: PCR primers and sequencing primers for detection of SNPs in the human HFE gene*	972442
PyroMark PCR Kit†	For 200 reactions: 2x PyroMark PCR Master Mix (includes HotStarTaq DNA Polymerase and optimized PyroMark Reaction Buffer containing 3 mM MgCl2 and dNTPs), 10x CoralLoad Concentrate, 5x Q-Solution, 25 mM MgCl2, and RNase-Free Water	978703
Accessories		
PyroMark Gold Q24 Reagents (5 x 24)	Nucleotides, enzyme, and substrate solutions, intended for use with PyroMark Q24	970802
PyroMark Gold Q96 reagents (5 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 ID	972804
PyroMark Gold Q96 reagents (6 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 MD in combination with the capillary dispensing tips (CDT)	972824
PyroMark Binding Buffer (200 ml)	Solution providing optimal conditions for immobilization of biotinylated DNA to streptavidin-coated Sepharose beads	979006
PyroMark Denaturation Sol. (500 ml)	Solution for use with the PyroMark Q24 Vacuum Workstation and PyroMark Q96 Vacuum Workstation for preparation of single stranded DNA template	979007
PyroMark Wash Buffer (conc., 200 ml)	Solution for use with the PyroMark Q24 Vacuum Workstation and PyroMark Q96 Vacuum Workstation to wash and neutralize the immobilized DNA	979008

* Not available in all countries; please inquire.

† Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
PyroMark Annealing Buffer (250 ml)	Solution providing optimal conditions for annealing of sequencing primer to DNA template	979009
PyroMark Q24 Plate (100)	24-well plate for samples, for use with PyroMark Q24; 100 plates in each package	979201
PyroMark Q24 Cartridge (3)	Reusable dispensing tool for nucleotide and reagent delivery, for use with PyroMark Q24	979202
PyroMark Q96 Plate Low (100)	For sample analysis of DNA template prepared with magnetic beads; for use with PyroMark Q96 ID	979002
PyroMark Q96 Sample Prep Thermoplate Low	Holder for placement of PyroMark Q96 Plate Low on heating block during annealing step prior to PyroMark Q96 ID analysis	9019070
PyroMark Q96 Cartridge (3)	For delivery of nucleotides and reagents for use with PyroMark Q96 ID	979004
PyroMark Q96 HS Plate (100)	For sample analysis of DNA template on PyroMark Q96 MD	979101
PyroMark Q96 HS Sample Prep Thermoplate	Holder for placement of PyroMark Q96 Plate on heating block during annealing step prior to PyroMark Q96 MD analysis	9019071
PyroMark Q96 HS Dispensing Tip Holder	Reusable holder for tips, nucleotide dispensing tips, and reagent dispensing tips, for use with PyroMark Q96 MD	9019075
PyroMark Q96 HS Capillary Tip Holder	Reusable holder for tips, nucleotide dispensing tips, and reagent dispensing tips, for use with PyroMark Q96 MD	9019076
PyroMark Q96 HS Reagent Tips (4)	Reusable tips (4 in each package); for dispensing reagents (RDTs); for use with PyroMark Q96 MD	979102
PyroMark Q96 HS Nucleotide Tips (8)	Reusable tips (8 in each package); for dispensing nucleotides (NDTs); for use with PyroMark Q96 MD	979103

Product	Contents	Cat. no.
PyroMark Q96 HS Capillary Tips (8)	Reusable capillary tips (8 in each package); for dispensing nucleotides (CDTs); for use with PyroMark Q96 MD	979104
Related products		
PyroMark Q24	Instrument, software, and installation for Pyrosequencing analysis	9001514
PyroMark Q24 Software 2.0	Analysis software, for laboratory use only	9019062
PyroMark Q96 ID	Instrument, for laboratory use only	9001525
PyroMark Q96 ID Software	Application software, for laboratory use only	9019083
PyroMark Q96 MD	Instrument, for laboratory use only	9019526
PyroMark Q96 MD Software	Application software, for laboratory use only	9019085
PyroMark Q24 Vacuum Workstation	For preparation of single stranded DNA template ready for sequencing by PyroMark Q24	Varies*
PyroMark Q96 Vacuum Workstation	For preparation of single stranded DNA template ready for sequencing by PyroMark Q96	Varies†
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PyroMark Q24 Validation Oligo	For performance check of the PyroMark Q24 system	979204

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* 9001518 (220 V); 9001516 (110 V); 9001519 (100 V).

† 9001529 (220 V); 9001528 (110 V); 9001740 (100 V).

Notes

Notes

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