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PyroMark Q24 User Manual



Sample & Assay Technologies

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1 Safety Information

Before using the PyroMark Q24 system, it is essential that you read this user manual carefully and pay particular attention to the safety information. The instructions and safety information in the user manual must be followed to ensure safe operation of the instrument and to maintain the instrument in a safe condition.


The following types of safety information appear throughout this manual.

<p>WARNING</p> 	<p>The term WARNING is used to inform you about situations that could result in personal injury to you or other persons. Details about these circumstances are given in a box like this one.</p>
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<p>CAUTION</p> 	<p>The term CAUTION is used to inform you about situations that could result in damage to the instrument or other equipment. Details about these circumstances are given in a box like this one.</p>
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The advice given in this manual is intended to supplement, not supersede, the normal safety requirements prevailing in the user's country.

1.1 Proper use

<p>WARNING</p> 	<p>Risk of personal injury and material damage ^[W1] Improper use of the PyroMark Q24 may cause personal injuries or damage to the instrument. The PyroMark Q24 must only be operated by qualified personnel who have been appropriately trained. Servicing of the PyroMark Q24 must only be performed by QIAGEN Field Service Specialists.</p>
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Safety Information


Perform the maintenance as described in Section 6. QIAGEN charges for repairs that are required due to incorrect maintenance.

WARNING 	Risk of personal injury and material damage [W2] The PyroMark Q24 is too heavy to be lifted by one person. To avoid personal injury or damage to the instrument, do not lift the instrument alone.
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WARNING 	Risk of personal injury and material damage [W3] Do not attempt to move the PyroMark Q24 during operation.
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1.2 Electrical safety

Disconnect the line power cord from the power outlet before servicing.


WARNING 	Electrical hazard [W4] Any interruption of the protective conductor (earth/ground lead) inside or outside the instrument or disconnection of the protective conductor terminal is likely to make the instrument dangerous. Intentional interruption is prohibited. Lethal voltages inside the instrument When the instrument is connected to line power, terminals may be live, and opening covers or removing parts is likely to expose live parts.
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To ensure satisfactory and safe operation of the PyroMark Q24, follow the advice below:

- The line power cord must be connected to a line power outlet that has a protective conductor (earth/ground).
- Keep mains plugs easily accessible in case the equipment needs to be disconnected quickly from mains power.
- Use only power supplies and cords supplied with the instrument.

1.3 Biological safety

When handling biological material, use safe laboratory procedures as outlined in publications such as *Biosafety in Microbiological and Biomedical Laboratories*, HHS (www.cdc.gov/od/ohs/biosfty/biosfty.htm).


<p>WARNING</p> 	<p>Biological materials [W5]</p> <p>Handle biological material with the greatest of care and in accordance with the required safety regulations. Always wear safety glasses, 2 pairs of 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 suitably trained and not exposed to hazardous levels of infectious agents as defined in the applicable Material Safety Data Sheets (MSDSs) or OSHA,* ACGIH,[†] or COSHH[‡] documents.</p> <p>Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.</p>
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* OSHA: Occupational Safety and Health Administration (United States of America).

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

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

1.4 Chemicals

WARNING 	Hazardous chemicals [W5] 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 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.
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* OSHA: Occupational Safety and Health Administration (United States of America).


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

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


1.5 Mechanical hazards


The lid of the PyroMark Q24 must remain closed during operation of the instrument. An audible warning signal will alert you if the lid is opened when it is not safe.

WARNING 	Moving parts [W6] To avoid contact with moving parts during operation of the PyroMark Q24, the instrument must be operated with the lid closed. Do not remove the cover panels since there are no user-serviceable parts inside. If there is a problem with the PyroMark Q24, contact QIAGEN Technical Services immediately.
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CAUTION 	Sharp needles [C1] Do not touch the sharp needles at the bottom of the reagent cartridge.
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1.6 Heat hazard



WARNING 	Hot surface [W7] The plate holder and the heating block can reach temperatures of up to 80°C (176°F). Avoid touching them when they are hot.
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WARNING 	Risk of overheating [W8] To ensure proper ventilation, maintain a minimum clearance of 10 cm (3.94 in.) at the sides and rear of the PyroMark Q24. Slits and openings that ensure the ventilation of the PyroMark Q24 must not be covered.
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1.7 Consumables




CAUTION 	Unsupported consumables [C2] Do not connect or use any consumables, accessories, or external equipment other than that specified.
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1.8 Symbols used on the PyroMark Q24

Symbol	Location	Language	Description
	Type plate on the back of the instrument	EN	CE mark
	Type plate on the back of the instrument	EN	Conforms to United States and Canadian safety standards

Safety Information

Symbol	Location	Language	Description
	Type plate on the back of the instrument	EN	FCC mark of the United States Federal Communications Commission
	Type plate on the back of the instrument	EN	C-Tick mark for Australia (supplier identification N17965)
	Type plate on the back of the instrument	EN	RoHS mark for China (the restriction of the use of certain hazardous substances in electrical and electronic equipment)
	Type plate on the back of the instrument	EN	WEEE mark for Europe
	All products	EN	Catalog or recorder number
	Reagents, solutions, cartridge	EN	Lot number (or batch code)
	Reagents, solutions, cartridge	EN	To be used by
	Reagents, solutions, cartridge	EN	Temperature limitation
	Instrument, VPW, reagents, cartridge	EN	Read the manual

Symbol	Location	Language	Description
	Type plate on the back of the instrument and all other products	EN	Manufacturer
	Inside the instrument	EN	Warning, consult user manual
	Denaturation Solution	EN	Irritant substance

Safety Information

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2 Introduction

Thank you for choosing the PyroMark Q24. We are confident it will become an integral part of your laboratory.

Before using the PyroMark Q24, it is essential that you read this user manual carefully and pay particular attention to the safety information. The instructions and safety information in the user manual must be followed to ensure safe operation of the instrument and to maintain the instrument in a safe condition.

2.1 About this user manual

This user manual provides information about the PyroMark Q24 in the following sections:

1. Safety Information
 2. Introduction
 3. General Description
 4. Installation Procedures
 5. Operating Procedures
 6. PyroMark Q24 Software
 7. Maintenance Procedures
 8. Troubleshooting
 9. Glossary
- Appendices

The appendices include the following:

- Technical data
- Methods and accessories
- Warranty terms.

2.2 General information

2.2.1 Technical assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Services 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 the PyroMark Q24 or QIAGEN products in general, 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/goto/TechSupportCenter or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

2.2.2 Policy statement

It is the policy of QIAGEN to improve products as new techniques and components become available. QIAGEN reserves the right to change specifications at any time.

In an effort to produce useful and appropriate documentation, we appreciate your comments on this user manual. Please contact QIAGEN Technical Services.

2.2.3 Version management

This document is the *PyroMark Q24 User Manual*, version 1.0.

2.3 Intended use of the PyroMark Q24

The PyroMark Q24 System is intended to be used to analyze up to 24 DNA templates in terms of (i) the level of methylation at multiple, individual CpG sites, (ii) the proportion of one or more alleles at one or more variable positions, or (iii) the genotype of one or more variable sites. Correct performance can only be ensured if the supplied user documentation is followed in combination with correct reagents.

The PyroMark Q24 system is intended for use by professional users, such as technicians and physicians trained in molecular biological techniques and the operation of the PyroMark Q24 system.

The PyroMark Q24 is intended to be used only in combination with QIAGEN kits indicated for use with the PyroMark Q24 and for applications described in the respective kit handbooks.

2.3.1 Requirements for PyroMark Q24 users

The table below covers the general level of competence and training necessary for transportation, installation, use, maintenance, and servicing of the PyroMark Q24.

Introduction

Task	Personnel	Training and experience
Delivery	No special requirements	No special requirements
Installation	QIAGEN Field Service Specialists only	
Routine use (running protocols)	Laboratory technicians or equivalent	Appropriately trained and experienced personnel familiar with use of computers and automation in general
Preventive maintenance	Laboratory technicians or equivalent	Appropriately trained and experienced personnel familiar with use of computers and automation in general
Servicing and annual preventive maintenance	QIAGEN Field Service Specialists only	

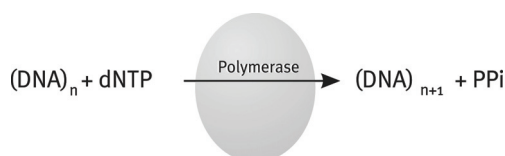
3 General Description

The PyroMark Q24 System is a system for detecting changes in specified variable positions in human DNA that may have clinical relevance using a technique called Pyrosequencing®. The instrument can analyze 24 samples simultaneously. An easy-to-use protocol is used to prepare samples.

3.1 Pyrosequencing principle

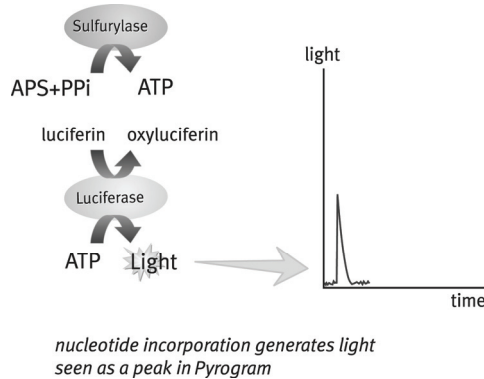
Pyrosequencing uses sequencing by synthesis for accurate and quantitative analysis of DNA sequences.

1. A sequencing primer is hybridized to a single-stranded, PCR-amplified DNA template.
2. The template is incubated with enzymes and substrates.
3. The first of four nucleotides is added to the reaction. If the nucleotide is complementary to the base in the template strand it will be incorporated into the DNA strand by the DNA polymerase.
4. Each incorporation event is accompanied by release of pyrophosphate (PPi) in an equimolar quantity to the amount of nucleotide incorporated.



5. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate.
6. This drives the conversion of luciferin to oxyluciferin by luciferase, generating visible light in amounts proportional to the amount of ATP. Light is detected using charged coupled devices (CCDs) and seen as a peak in the Pyrogram®. Each light signal is proportional to the number of nucleotides incorporated.

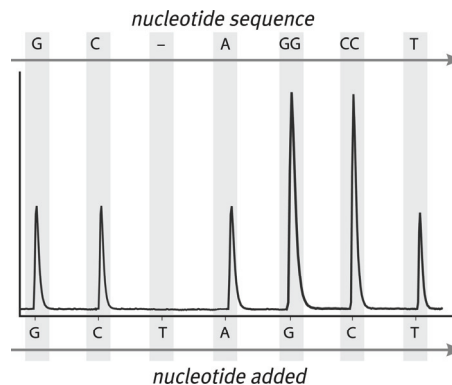
General Description



7. Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.



8. Nucleotides are added one at a time.
Note: Deoxyadenosine alfa-thio triphosphate (dATP α S) is used instead of natural deoxyadenosine triphosphate (dATP) since it is used efficiently by the DNA polymerase, but not recognized by the luciferase.
9. As the process continues, the complementary sequence is built up and the nucleotide sequence is determined from the signal peak in the Pyrogram.

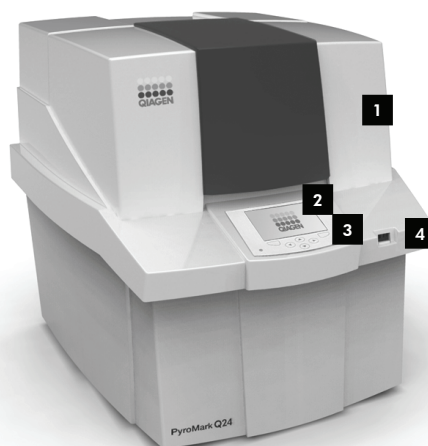


3.2 PyroMark Q24 principle

The PyroMark Q24 performs DNA sequencing using the Pyrosequencing technique.

1. The PyroMark plate containing the samples is placed on the heating block and the PyroMark Q24 reagent cartridge is filled with reagents and placed in the dispensing unit.
2. The USB stick containing the run file created in PyroMark Q24 software is inserted into the USB port at the front of the instrument. The run is then started.
3. The dispensing unit pressure, mixer speed, and temperatures of the heating block, process chamber lid and coolant liquid are adjusted to preset levels.
4. Enzyme and substrate mixtures are dispensed into the plate, to ensure that the dispensation capillaries are flushed and filled with solution.
5. Enzyme mixture and then substrate mixture are dispensed into all wells used.
6. The pressure in the dispensing unit is increased.
7. Nucleotides are dispensed into the plate before being dispensed into the wells used according to the dispensation order set. Nucleotides are added in sequence and 65 seconds elapses between the additions of each nucleotide to ensure all enzymatic reactions are completed.
8. The instrument collects data simultaneously from all wells using 24 CCDs located under the heating block. Data is stored on the instrument.
9. After the run, data is automatically stored on the USB stick. If the USB stick has been removed during a run, data can be retrieved manually from the instrument using the USB stick.

3.3 External features of the PyroMark Q24



The PyroMark Q24.

- | | | | |
|----------|----------------|----------|--------------|
| 1 | Instrument lid | 3 | Menu buttons |
| 2 | Screen | 4 | USB port |

3.4 Process chamber



Heating block.

The process chamber contains a heating block that maintains the correct temperature of the plate and its contents. If the room temperature is too high, the heating block is cooled by PyroMark Q24 Cooling Device (preinstalled).

Data are collected from all the wells simultaneously by 24 CCDs underneath the heating block. In wells where there is a positive reaction with the added nucleotide, light is emitted giving rise to a peak in Pyrogram. To enable fast mixing of samples and reagents in the plate, the heating block inside the process chamber is constantly vibrated during the run.

3.5 Dispensing unit



Dispensing unit.

A reagent cartridge (PyroMark Q24 Cartridge) filled with the required volumes of reagents is inserted into the dispensing unit. The instrument starts dispensing reagents when the pressure in the dispensing unit, the speed of the mixer, and the temperatures of the heating block, process chamber lid, and the coolant liquid reach their preset levels (this may take several minutes). During the run, the reagent cartridge is positioned over each well in the plate and reagents are dispensed in a zigzag fashion by a pneumatic system.

3.6 Computer and software

The PyroMark Q24 is shipped with the PyroMark Q24 Software.

The computer used for setup of runs and data analysis should have the following as minimum specifications:

General Description

- Microsoft® Windows® XP or Windows Vista (English version) Operating System
- Pentium® IV processor (3 GHz) or higher
- 100 MB free hard drive capacity
- 1 GB RAM
- Monitor with 1280 x 1024 pixels
- Graphics card supporting the resolution of the monitor
- Pointer device (mouse or similar)
- USB-port and CD-ROM interfaces.

4 Installation Procedures

4.1 Instrument delivery and installation

The unpacking and installation of the PyroMark Q24 is carried out by a certified QIAGEN Field Service Specialist. A member of your group who is familiar with laboratory and computer equipment should be present during the installation.

The following items are delivered:

- *PyroMark Q24 User Manual* in PDF format on accompanying CD-ROM
- *PyroMark Q24 Software Guide* in PDF format on accompanying CD-ROM
- USB stick

4.2 Requirements

Site

The PyroMark Q24 and PyroMark Q24 Vacuum Workstation must be located out of direct sunlight, away from heat sources, and away from sources of vibration and electrical interference. Refer to Appendix A for the operating conditions (temperature and humidity). The site of installation should be free of excessive drafts, excessive moisture, excessive dust, and not subject to large temperature fluctuations.

Refer to Appendix A for the weight and dimensions of the PyroMark Q24 and PyroMark Q24 Vacuum Workstation.

Ensure that the workbench is level, dry, clean, vibration-proof, and has additional space for accessories.


Approximately 70 cm (27 in.) clearance above the workbench is required to accommodate the PyroMark Q24 with the lid open. Allow at least 10 cm (4 in.) of free space behind the instrument for calibration.

The PyroMark Q24 must be placed within approximately 1.5 m (59 in.) of a properly grounded (earthed) AC power

Installation Procedures

outlet. The power line to the instrument should be voltage regulated and surge protected.

Note: We recommend plugging the instrument directly into its own wall socket and not to share the socket with other lab equipment. To achieve proper capillary electrophoresis separation, do not place the PyroMark Q24 on a vibrating surface or near vibrating objects.

WARNING 	Risk of overheating [W8] To ensure proper ventilation, maintain a minimum clearance of 10 cm at the sides and rear of the PyroMark Q24. Slits and openings that ensure the ventilation of the PyroMark Q24 must not be covered.
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Power requirements

The PyroMark Q24 operates at:

- 100–240 V AC, 47–63 Hz, 1.1–0.45 A. Power consumption: Maximum 160 Watt

The PyroMark Q24 Vacuum Workstation operates at:

- 100 V AC, 50/60 Hz, 1.7/1.4 A (power consumption: Maximum 25 Watt)
- 115 V AC, 60 Hz, 1.5 A (power consumption: Maximum 25 Watt)
- 230 V AC, 50 Hz, 0.6 A (power consumption: Maximum 25 Watt)

Make sure that the voltage rating of the PyroMark Q24 is compatible with the AC voltage available at the installation site. Mains supply voltage fluctuations are not to exceed 10% of nominal supply voltages.

Grounding requirements

To protect operating personnel, the PyroMark Q24 must be correctly grounded (earthed). The instrument is equipped with a 3-conductor AC power cord. To preserve this protection feature, do not operate the instrument from an AC power outlet that has no ground (earth) connection.

5 Operating Procedures

This section describes how to operate the PyroMark Q24 system.

Before proceeding, we recommend that you familiarize yourself with the features of the PyroMark Q24 by referring to Section 3.3.

5.1 Instrument administration

5.1.1 Setting date and time

Setting the date and time correctly ensures an accurate date and time stamp in the instrument and run logs and the analysis reports. Set the date and time as follows:

1. When the instrument is not performing a run, select "Administration" in the main menu.
2. Select "Date and Time" and press "OK".
3. Select the parameter you want to edit using ◀ and ▶ screen buttons.
4. Edit the selected parameter using the ▲ and ▼ screen buttons.
5. To edit further parameters, repeat steps 3 and 4.
6. To save the change(s), press "Set".

5.1.2 Copying unsaved runs

If the USB stick is removed before the run is finished, retrieve the run data from the instrument as follows:

1. When the instrument is not processing, insert the USB stick into the USB port at the front of the instrument.
2. Select "Administration" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
3. Select "Copy Unsaved Runs" and press "OK".
4. Select the run file for retrieval (using the ▲ and ▼ screen buttons) and press "Select".
5. When the instrument confirms that the run file has been saved to the USB stick, press "Close".

6. Remove the USB stick.

5.1.3 Copying recently saved runs

Copies of run files are stored on the instrument provided there is enough free space in the internal USB.

Note: When space becomes insufficient, the run files are deleted in chronological order. Files that have never been saved to a USB stick will not be deleted.

Copy recently saved runs as follows:

1. When the instrument is not performing a run, insert the USB stick into the USB port at the front of the instrument.
2. Select "Administration" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
3. Select "Copy Recently Saved Runs" and press "OK".
4. Select the run file for retrieval (using the ▲ and ▼ screen buttons) and press "Select".
5. When the instrument confirms that the run file has been saved to the USB stick, press "Close".
6. Remove the USB stick.

5.1.4 Copying log files

If you need to send log files to QIAGEN Technical Services, copy files as follows:

1. When the instrument is not performing a run, insert one of the USB sticks (supplied with the system) into the USB port at the front of the instrument.
2. Select "Administration" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
3. Select "Copy Log Files" and press "OK".
4. When the instrument confirms that the log files have been saved to the USB stick, press "Close".
5. Remove the USB stick.

5.1.5 Extracting damaged runs

If runs are damaged (e.g., if the instrument was turned off during a run), extract run files as follows:

1. When the instrument is not performing a run, insert one of the USB sticks (supplied with the system) into the USB port at the front of the instrument.
2. Select "Administration" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
3. Select "Extract Damaged Runs" and press "OK".
4. When the instrument confirms that the run files have been saved to the USB stick, press "Close".
5. Remove the USB stick.

5.1.6 Backup of PyroMark Q24 files

The data generated by PyroMark Q24 software is stored on the computer as files with the following suffixes:

- ***.pyrorun** (run files)
- ***.pyrosetup** (assay files).

Data backup should be performed frequently. This can be done by copying PyroMark Q24 files (***.pyrorun** and ***.pyrosetup**) to another location. The alternative location should be another physical drive or permanent medium.

For more information about backup, contact your system administrator.

5.1.7 Viewing acknowledgements, version, legal, and contact information

View acknowledgements, legal information, software and hardware versions or contact information as follows:

1. Select "About" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
2. Select the information you want to view and press "OK".

5.1.8 Upgrading the instrument software

If you have received a software upgrade from QIAGEN, upgrade the software as follows:

1. Save the upgrade files on one of the USB sticks supplied with the system. The files should be saved in a folder named "Upgrade" in the root directory of the USB stick.
2. When the instrument is not running, insert the USB stick into the USB port at the front of the instrument. Do not remove it until the upgrade is completed.
3. Select "Administration" in the main menu (using the ▲ and ▼ screen buttons) and click "OK".
4. Select "Upgrade Software" and click "OK".
5. Follow the instructions on the screen.

5.1.9 Run an external application

The "Run External Application" menu option is used for service applications. Only run a service application when instructed by QIAGEN Technical Services.

5.2 Setting up a run

Before setting up a run, we recommend that you familiarize yourself with the safety information by referring to Section 1.


Detailed instructions on setting up a run can also be found in the online help, by pressing the F1 key when in a dialog box or window in the software.

5.2.1 Starting the PyroMark software

In the Windows "Start" menu, select "All Programs/Biotage/PyroMark Q24".

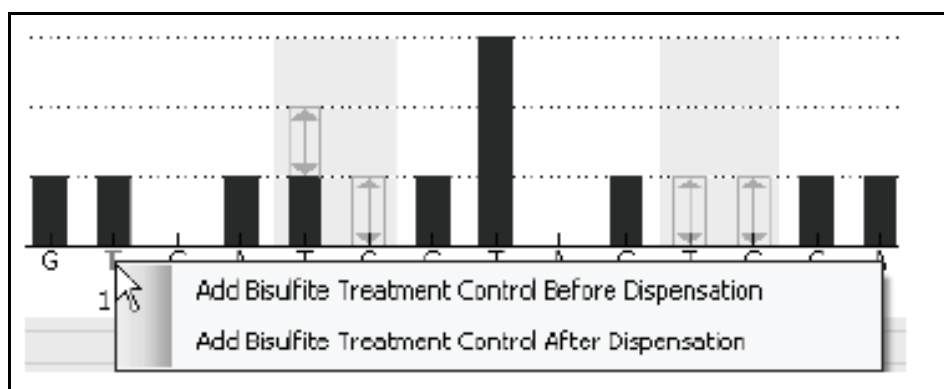
Context-sensitive help can be accessed at any time by pressing the F1 key.

5.2.2 Setting up an assay

1. In the shortcut browser, right-click the folder you want to place the assay file in and select "New Assay" followed by "AQ Assay" or "CpG Assay" from the context menu. To add a shortcut to a folder in the shortcut browser, click "Add Folder Shortcut".
2. Enter the file name and press "Enter".
3. Type or paste the "Sequence to Analyze".
4. Click the "Generate Dispensation Order" button.
5. Click  in the toolbar.
The more frequently a file is saved, the more information is recovered if there is a power failure or problem while the file is open. PyroMark Q24 files should be backed-up frequently.
6. Before running your samples, validate your assay using reference samples; see Appendix B, page B-1.

Optional

If desired, enter a note about the assay and set up the variable positions at the "Variable Positions" tab. If creating a CpG assay, we recommend bisulfite treatment controls are added. To do this, left-click a bold, orange **T** or **A** in the histogram, preferably at the beginning of the sequence.



Note: In the sequence before bisulfite treatment (can be entered in the assay), check if the suggested bisulfite controls are Cs converted to **Ts** (read as Gs and **As** in a reverse assay) or not.

5.2.3 Setting up a run



1. In the shortcut browser, right-click the folder you want to place the run file in and select "New Run" from the context menu.
To add a shortcut to a folder in the shortcut browser, click "Add Folder Shortcut".
2. Enter the file name and press "Enter".
3. Select "Instrument Method"; see the instructions supplied with the used reagents and cartridge.
4. To set up the plate, add an assay to each well used, e.g., drag an assay in the shortcut browser to a well or a selection of wells.

A well is colored according to the assay loaded into the well.

Plate Setup				
	1	2	3	4
A	CpG Assay 1	CpG Assay 2	AQ Assay 1	AQ Assay 2

5. To enter a sample ID or note, select the cell and enter the text. A selected cell is highlighted with a blue background color.

Assay Name	Assay Name
Sample ID	Sample ID
Note	Note

6. Click  in the toolbar.
The more frequently a file is saved, the more information is recovered if there is a power failure or problem while the file is open. PyroMark Q24 files should be backed-up frequently.
7. Print a list of required volumes of reagents and the plate setup; select "Pre Run Information" from the "Tools" menu and, when the report appears, click .
8. Close the run file and copy it to one of the USB sticks supplied with the system.

Optional

If desired, enter the "Reagent ID" (i.e., the lot number for PyroMark Q24 Gold Reagents), a "Plate ID", a "Bar code" for the plate, and a "Run Note".

Further information

There are several ways to set up a plate. For example, it is possible to import and paste a sample layout defined in a text file, and drag-copy and increment a sample ID (if the last part of the entered sample ID is a number). For more information, see the online help. Context-sensitive help is accessed by pressing the F1 key when in a dialog box or window in the PyroMark Q24 Software.

Note: To base your run on a previous run, right-click the processed run file in the shortcut browser and select "Copy and Rerun" from the context menu. The process and analysis data will not be copied.

5.3 Sample preparation

Samples to be analyzed using the PyroMark Q24 should be prepared according to the instructions below.

The following equipment and reagents are required for sample preparation. All reagents and solutions should be at room temperature (15–25°C) before starting. All steps are performed at room temperature unless otherwise stated.

Equipment and reagents to be supplied by the user

- PyroMark Q24 Vacuum Workstation
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80–85°C
- PyroMark Q24 Plate (cat. no. 979201)
- 24-well PCR plate or strips
- Strip caps
- Streptavidin Sepharose High Performance (34 μ m, 5 ml, GE Healthcare)
- Sequencing primer
- Nuclease-free Water (cat. no. 129114)

- Ethanol (70%)
- PyroMark Binding Buffer (cat. no. 979006)
- PyroMark Denaturation Solution (cat. no. 979007)
QIAGEN
- PyroMark Wash Buffer concentrate (cat. no. 979008)
- PyroMark Annealing Buffer (cat. no. 979009)

5.3.1 **PyroMark Q24 Vacuum Workstation function test**

Before using the PyroMark Q24 Vacuum Workstation, check that the filter probes are working by performing the function test as follows:

1. Add 100 μ l nuclease-free water to each well of a PCR plate.
2. Fill a trough with 70 ml nuclease-free water.
3. Start the vacuum pump
4. Apply vacuum to the vacuum prep tool by opening the vacuum switch.
5. Lower the filter probes into the trough. Keep them in position for approximately 20 s. Ensure that the water is transferred to the waste container, i.e., that vacuum has been applied. If this has not happened, check the connections.
6. Lower the filter probes into the PCR plate and check that the water is aspirated evenly across all wells and that all wells are empty after a maximum of 10 s.
7. If the wells are not empty after 10 s, repeat the procedure from step 1. If the function test fails twice, replace the filter probes (see Section 6.3.2).

5.3.2 **DNA amplification**


Amplify the DNA by PCR using one of the primers biotinylated. To receive valid analysis data, see Appendix B, page B-1.

5.3.3 **Immobilizing the PCR product to beads**

Biotinylated PCR products are immobilized on streptavidin-coated Sepharose beads (Streptavidin Sepharose High Performance, GE Healthcare).

1. Gently shake the bottle with streptavidin-coated Sepharose beads from side to side until a homogenous solution is obtained.
2. Mix the total amount of streptavidin-coated Sepharose beads (2 μ l per sample) and Binding Buffer (40 μ l per sample) in a tube. Add 18–28 μ l nuclease-free water to make the total volume 80 μ l per well.
Example: If using 15 μ l of PCR product, 2 μ l of beads, and 40 μ l of Binding Buffer, 23 μ l of nuclease-free water must be added.
3. Add the solution prepared in step 2 to the PCR plate (or the strips).
4. Add 10–20 μ l of a well optimized, biotinylated PCR product to each well of a 24-well PCR plate or strips according to the plate setup (see Section 5.2.3).
Note: The total volume per well should be 80 μ l.
5. Seal the PCR plate (or the strips) using strip caps. Ensure that no leakage is possible between the wells.
6. Agitate the PCR plate (or strips) constantly for at least 5–10 min using a mixer (1400 rpm).
Note: Sepharose beads sediment quickly and capturing of beads must take place immediately once the agitation is complete.
Note: During immobilization, prepare the vacuum workstation for the sample preparation (steps 1–8 in Section 5.3.4).

5.3.4 Separation of DNA strands and release of samples in the PyroMark Q24 plate

WARNING 	Hazardous chemicals [W5] 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 Material Safety Data Sheets (MSDSs) or OSHA,* ACGIH, [†] or COSHH [‡] documents. For more information, visit www.qiagen.com/support/msds.aspx . Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.
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* OSHA: Occupational Safety and Health Administration (United States of America).

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

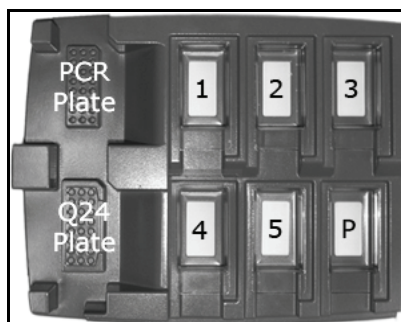
[‡] COSHH: Control of Substances Hazardous to Health (United Kingdom).

Procedure

1. Ensure that the PyroMark Q24 Vacuum Workstation has been assembled correctly and securely. The mains plug should be easily accessible in case the vacuum pump needs to be disconnected quickly from the mains power.
Note: Perform the function test to ensure that the filter probes are working properly (see Section 5.3.1). All probes should be replaced after preparation of approximately 100 plates.
Note: Check and empty the waste container if necessary (see Section 5.6).
2. Fill five separate troughs (supplied with the PyroMark Q24 Vacuum Workstation) as follows:
 - Approximately 50 ml ethanol (70%) (1)
 - Approximately 40 ml Denaturation Solution (2)
 - Approximately 50 ml Washing Buffer (3)

- Approximately 50 ml nuclease-free water (4)
- Approximately 70 ml nuclease-free water (5).

A suggested setup is shown below. Refill the troughs to these levels whenever necessary.



3. Switch on the vacuum pump.
4. Apply vacuum to the tool by opening the vacuum switch.
5. Wash the filter probes by lowering the probes into nuclease-free water (trough 5). Flush the probes with 70 ml nuclease-free water.
Ensure that the water is being transferred to the waste container. If it is not, ensure the tubing is connected correctly and is not broken. Broken tubing should be replaced, see Section 6.3.4.
Ensure that the waste filter is dry. If the filter is wet, it should be replaced, see Section 6.3.5.
6. Refill trough 5 with 70 ml nuclease-free water.
7. Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.
8. Add 0.3 μ M sequencing primer in 25 μ l Annealing Buffer to each well of a PyroMark Q24 Plate that is to be used.
Note: Use one of the supplied PyroMark Q24 Plate Holders as support when preparing and moving the plate.
9. Place the PCR plate and PyroMark Q24 Plate on the worktable. Ensure that the plate is in the same orientation as when samples were loaded.
10. Apply vacuum to the tool by opening the vacuum switch.
11. Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized

Operating Procedures

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.

12. Ensure that all liquid is aspirated from the wells and that all beads have been captured onto the filter probe tips.

Note: If the wells still contain liquid or white beads remain, the filter probes may need replacing, see Section 6.3.2.


13. Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.
14. Transfer the tool to the trough containing Denaturation Solution (trough 2). Flush the filter probes for 5 s.
15. Transfer the tool to the trough containing Washing Buffer (trough 3). Flush the filter probes for 10 s.
16. Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes (see image).



17. Whilst the tool is held over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).
18. Release the beads in the plate containing 0.3 μ M sequencing primer in 25 μ l Annealing Buffer, by shaking the tool gently from side to side. Allow the filter probes to rest on the bottom of the wells.
19. Transfer the tool to the trough containing nuclease-free water (trough 4) and agitate the tool for 10 s.


20. Wash the filter probes by lowering the probes into nuclease-free water (trough 5) and applying vacuum. Flush the probes with 70 ml nuclease-free water.
21. Raise the tool to beyond 90° vertical for 5 s, to drain liquid from the filter probes.
22. Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.
23. If more than one plate is prepared at once, refill the troughs (step 2) and repeat the procedure from step 8.
24. Turn off the vacuum pump.
25. At the end of a working day, liquid waste and any remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage, see Section 5.6.

5.3.5 Annealing of samples to sequencing primer

WARNING 	Hot surface [W7] The plate holder and the heating block can reach temperatures of up to 80°C (176°F). Avoid touching them when they are hot.
---	---

1. Heat the PyroMark Q24 Plate with the samples at 80°C for 2 min using the PyroMark Q24 Plate Holder supplied with the vacuum workstation and a heating block.
2. Let the samples cool to room temperature (15–25°C) for at least 5 min. The plate can now be processed in the PyroMark Q24.

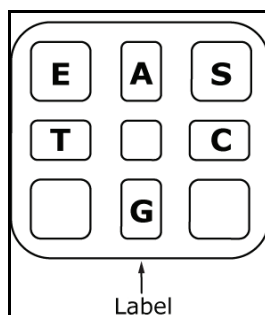
5.4 Preparation of PyroMark Q24 Gold Reagents

CAUTION 	Sharp needles [C1] Do not touch the sharp needles at the bottom of the reagent cartridge.
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1. Open the PyroMark Q24 Gold Reagents box and remove the vials containing freeze-dried enzyme and substrate mixtures, and the vials containing nucleotides.
2. Reconstitute the volumes of reagents required and fill PyroMark Q24 Cartridge according to the instructions supplied with the reagents.

Note: The required volumes of reagents are listed in the Pre Run Information report, see Section 5.2.3.

Note: Ensure any reused reagent cartridges are cleaned thoroughly, according to the instructions in Section 5.5.5. We recommend that the reagent cartridge is used a maximum of 30 times. If the reagent cartridge has not been used for an extended period of time (e.g., it has been stored), check that it can be used for analysis by performing the function test (steps 4–6 in Section 5.5.5).



PyroMark Q24 Cartridge.

5.5 Processing a run on the PyroMark Q24

The lid of the PyroMark Q24 must remain closed during operation of the instrument. An audible warning signal will alert you if the lid is opened when it is not safe.

WARNING




Moving parts

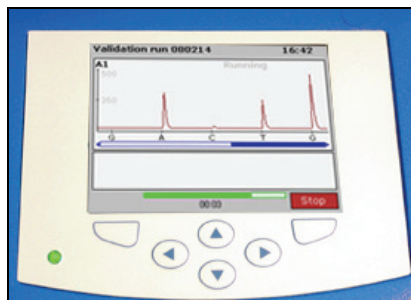
[W6]

To avoid contact with moving parts during operation of the PyroMark Q24, the instrument must be operated with the hood closed.

Do not remove the cover panels since there are no user-serviceable parts inside. If there is a problem with the PyroMark Q24, contact QIAGEN Technical Services immediately.

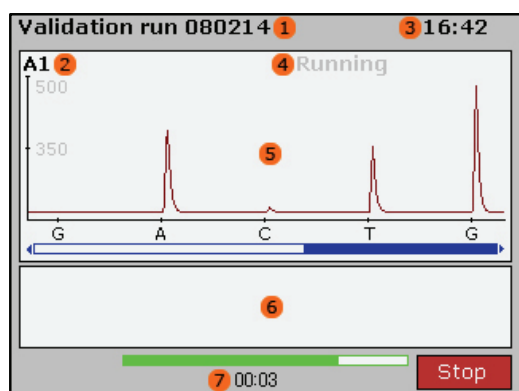
<p>CAUTION</p> 	<p>Sharp needles</p> <p>Do not touch the sharp needles at the bottom of the reagent cartridge.</p>	<p>[C1]</p>
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5.5.1 Instrument software



The instrument is controlled via the six buttons underneath the screen.

Runs are started and monitored through the software provided with the instrument. During processing of a run, the software displays the following information:



1. Run name
2. Selected well
3. Current time
4. Instrument status
5. Pyrogram
6. Warning messages
7. Estimated remaining time (hh:mm)

5.5.2 Starting the instrument

1. Before switching on the instrument, ensure that the mains plugs are connected to properly grounded (earthed) mains outlets with the correct voltage and frequency and that mains plugs are easily accessible in case the equipment needs to be disconnected quickly from mains power.
2. Switch on the instrument. The power switch is located at the rear of the instrument.

5.5.3 Starting the run

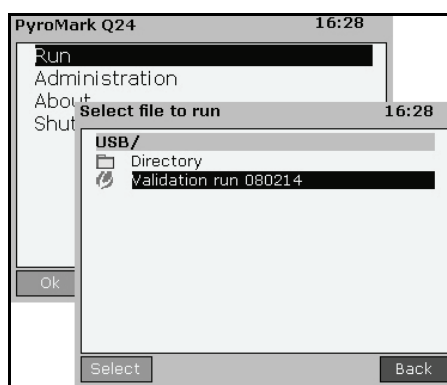
Load the reagent cartridge and the plate:

1. When the instrument is not processing, open the instrument lid. An audible warning signal will alert you if the lid is opened when it is not safe.
2. 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.
3. Ensure the line is visible in front of the cartridge and close the gate.
4. Open the plate-holding frame and place the plate on the heating block.
5. Close the plate-holding frame and the instrument lid.



Select the run file and start the run:

1. Insert the USB stick (containing the run file) into the USB port at the front of the instrument. Do not remove it until the run is finished.
2. Select "Run" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
3. 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".
4. When the run file is selected, press "Select" to start the run.



5.5.4 Monitoring the run

The instrument will start dispensing reagents when the pressure in the dispensing unit, the speed of the mixer, and the temperatures of the heating block, process chamber lid, and the coolant liquid have reached their preset levels.

Instrument status

The instrument status is displayed in the top right-hand corner.

Environment	Waiting for the pressure in the dispensing unit, the speed of the mixer, and the temperatures of the heating block, process chamber lid, and the coolant liquid to reach their preset levels (may take several minutes).
Priming	Priming the needles of the reagent cartridge to remove any air bubbles.
Running	The enzyme mix and substrate mix are dispensed to all wells used. Then the nucleotides are dispensed to the wells according to their dispensation order, which is defined in the assay file.
Stopped	The run has been aborted.
Saving	The run data is transferred to the USB stick. Do not remove the USB stick until the instrument confirms that the run file has been saved.
Finished	The run has been completed and the run data have been transferred to the USB stick.

Pyrogram and warnings

The run name and selected well are displayed in the top left corner. To select another well, use the ▲ and ▼ screen buttons.

Any instrument warnings are displayed below the Pyrogram area (the three latest warnings are displayed). For suggested actions, see Section 7.

Abort the run

To abort the run, press "Stop".

5.5.5 After the run

1. When the instrument confirms that the run file has been saved to the USB stick, press "Close".
2. Remove the USB stick.
3. Open the instrument lid.
4. Open the cartridge gate and remove the reagent cartridge by lifting it up and pulling it out.
5. Close the gate.
6. Open the plate-holding frame and remove the plate from the heating block.
7. Close the plate-holding frame and the instrument lid.
8. Discard the plate.
9. Clean the reagent cartridge according to the instructions in Section 5.5.5.
10. If this was the last run for the day, follow the instructions in Section 5.6.

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

Cleaning and testing the reagent cartridge

If the reagent cartridge is to be reused, clean it immediately after use and ensure that it can be used for analysis. It is recommended that the reagent cartridge is used a maximum of 30 times.

Note: Handle the needles of the reagent cartridge with care. Small particles and fibers may obstruct the needles.

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

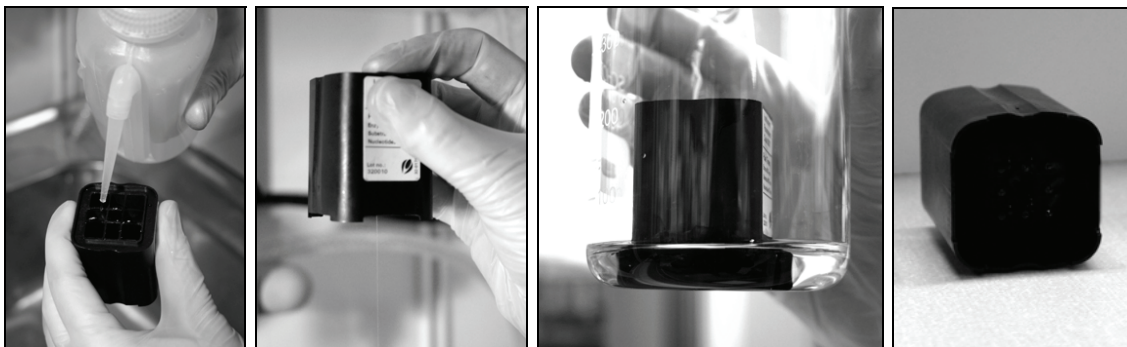
The following items are required:

- Powder-free gloves
- Nuclease-free Water (cat. no. 129114)
- Beaker (not always required)
- Lint-free tissues.

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To clean and check that the reagent cartridge can be used for analysis:

1. Discard remaining solutions.
2. Rinse the reagent cartridge 4 times with nuclease-free water.
3. Spray the outside of the needles using nuclease-free water.
4. Rinse the needles by completely filling the compartments with nuclease-free water.
Hold the reagent cartridge over a sink while pressing firmly on top of each compartment with a finger.
5. Check that the needle is clear.
A jet of water should squirt out from the tip of the needle. If a needle is blocked, e.g., if the reagent cartridge has been left overnight without cleaning, fill the compartments with nuclease-free water and immerse the cartridge in a beaker of nuclease-free water that covers the needles. Leave the reagent cartridge in the beaker for one hour, rinse it, and repeat steps 4 and 5.
6. Check that the jet of water is straight in the direction of the needle (not angled). If angled, refill the compartment with nuclease-free water and repeat. If the test fails, discard the reagent cartridge.
7. When all needles have been rinsed and tested, discard the water and leave the reagent cartridge to dry on its side on a lint-free tissue.
8. When the reagent cartridge is dry, store the cartridge in a dust-free place.



5.5.6 Analyzing the run

Detailed instructions for analyzing the run are available in the online help (press the F1 key when in a dialog box or window in the software).

1. Move the processed run file from the USB stick to a computer running PyroMark Q24 Software.
2. Open the run file by double-clicking the run file (✓) in the shortcut browser.
To add a shortcut to a folder in the shortcut browser, click "Add Folder Shortcut"
3. At the "Overview" tab, either analyze all wells or a selection of wells with a valid analysis setup for the selected analysis mode.



Analysis modes

AQ assays are analyzed in the AQ mode and CpG assays are analyzed in the CpG mode. SNP genotyping can be accessed through the AQ mode. To toggle between the modes, select "AQ" or "CpG" in the toolbar.

Note: How the analysis is performed can be modified using the "Analysis Setup" tab.

5.5.7 Viewing the analysis results

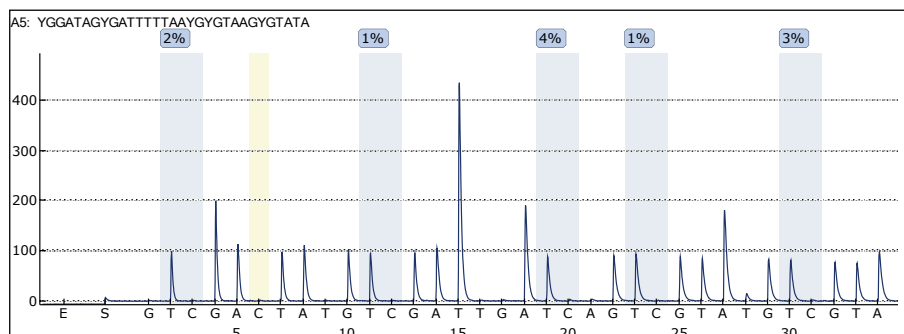
By selecting an analyzed well using the "Overview" tab, the following information is shown:

Well information

Assay name, sample ID, note, and any analysis warnings are listed in the "Well Information" area.

Pyrogram

The analysis results (the allele frequencies or the methylation percentages) are displayed above the variable positions in Pyrogram, for example 96%.

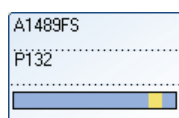


Variable positions are highlighted with a blue-gray background color, and bisulfite treatment controls with a light yellow background color.

Quality assessments

The quality assessments for the variable positions are displayed by:

- Quality bars in the plate overview at the "Overview" tab
- The background color of the analysis results in Pyrogram



C: 44% A: 93%
T: 56% T: 7%

□ Off-white: Not analyzed*

■ Blue: Passed




■ Yellow: Check

■ Red: Failed

* Either analysis is not supported by the software (e.g., SNP when in the CpG mode) or the variable position has been deselected by the user.

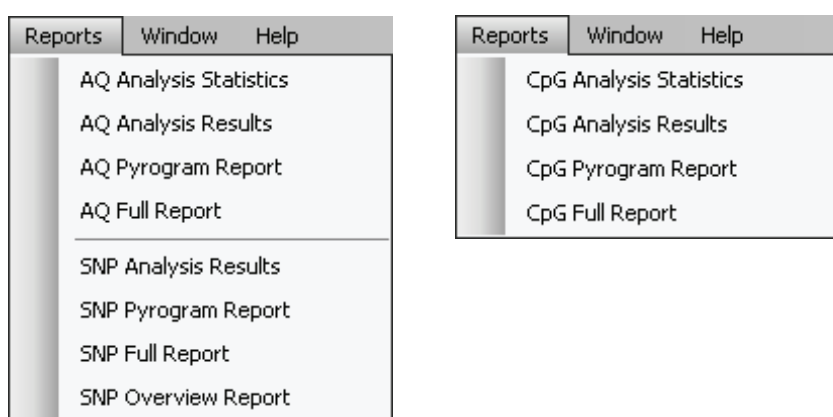
Methylation levels

When in the CpG mode, a methylation bar at the Overview tab shows the methylation level for each CpG site in the well.

-  Light green: Below the defined range
-  Green: Within the defined range
-  Dark green: Above the defined range

5.5.8 Analysis reports

To generate a report, select the desired report from the "Reports" menu. For more information about the reports, see the online help.



In order to view the Full, Pyrogram, and SNP Overview reports, you must have Adobe Acrobat Reader installed on your computer. Adobe Acrobat Reader is available on the PyroMark Q24 Software CD but can also be downloaded at www.adobe.com.

Context-sensitive help can be accessed by pressing the F1 key when in a dialog or window in the PyroMark Q24 Software.


5.6 Finishing work and shutting down

5.6.1 Shutting down the instrument

1. When the instrument is not performing a run, select "Shutdown" from the main menu (using the ▲ and ▼ screen buttons) and press "OK".

2. When the message "It is now safe to turn off the instrument" appears, switch off the instrument. The power switch is located at the rear of the instrument.

5.6.2 Emptying the waste container and troughs

WARNING 	Hazardous chemicals [W5] 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 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.
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* OSHA: Occupational Safety and Health Administration (United States of America).

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

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

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

The following item is required:

- Nuclease-free Water (cat. no. 129114).

Procedure:

1. Ensure that no vacuum is applied to the vacuum prep tool, i.e., 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 nuclease-free water, or replace them, if necessary.
4. Empty the waste container.

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

5. If the vacuum workstation must be cleaned (e.g., dust or spillage), follow the instructions in Section 6.3.1.

5.6.3 Checking the instrument

Check the instrument for dust and spillage. If the instrument needs to be cleaned, follow the instructions in Section 6.2.1.

At the rear of the instrument, press the light button and check that the coolant level is visible in the window. If is not, contact QIAGEN Technical Services.

Operating Procedures

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6 Maintenance Procedures

The following maintenance procedures must be carried out to ensure reliable operation of the PyroMark Q24:

- Regular performance checks
- Cleaning of the instrument

Following these procedures ensures that the PyroMark Q24 is free of dust and liquid spills.

Before undertaking maintenance procedures, we recommend that you familiarize yourself with the safety information by referring to Section 1.

Important: Disconnect the instrument from mains power before cleaning.

Servicing

The PyroMark Q24 is supplied with a warranty that lasts for 1 year, beginning on the date of shipment. The warranty includes all repairs due to mechanical breakdown.

Application development, software upgrades, accessories, and disposable items are not included in the warranty.

QIAGEN offers comprehensive Service Support Agreements, including Warranty Extensions, Full Cover Support Agreements, and instrument/application training, including on-site installation and annual preventative maintenance. Service Support Agreements maximize productivity and ensure high performance from your instrument. In addition, service histories are fully documented and all parts are certified and guaranteed.

Contact your local QIAGEN Field Service Specialist or your local distributor for more information about flexible Service Support Agreements from QIAGEN.

6.1 Checking the performance of the PyroMark Q24

Check that PyroMark Q24 System is functioning according to specifications by measuring imprecision, bias, and linearity using PyroMark Q24 Validation Oligo.

Perform the validation according to the instructions supplied with the product. To order PyroMark Q24 Validation Oligo, please contact QIAGEN.

6.2 Maintenance of the PyroMark Q24

6.2.1 Cleaning the instrument

If the instrument has been contaminated by dust and spillage, clean it according to the instructions below.

Important points before starting:

- Avoid harsh cleaners and chemicals, and getting moisture inside the instrument
- The cleaning liquid must be applied to the cloth only
- Do not use any organic solvent or detergent other than ethanol when cleaning the screen.

The following items are required:

- Ethanol (70%)
- Water
- Mild soap (if necessary)
- Clean, non-abrasive, lint-free cloths.

Procedure:

1. When the instrument is not processing, select "Shutdown" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
2. When the message "It is now safe to turn off the instrument" appears, switch off the instrument. The power switch is located at the rear of the instrument.
3. Disconnect the instrument from the mains power. There are two mains plugs.
4. Open the instrument lid.

5. Clean the area around the dispensing unit, the process chamber, and the heating block using a clean, lint-free cloth lightly moistened with 70% ethanol.
6. Clean the screen by wiping with a clean, non-abrasive, lint-free cloth lightly moistened with water.
If this does not clean the screen properly, apply a small amount of 70% ethanol to the cloth. Do not allow ethanol to soak into the gaps around the screen protection.
7. If necessary, clean the exterior of the instrument using a clean, lint-free cloth, lightly moistened with water. If required, a small amount of mild soap may also be used.
8. After cleaning, wipe the surfaces dry with a clean, dry, non-abrasive, lint-free cloth.
9. Reconnect the instrument to the mains power.

6.2.2 Cleaning the heating block and light guides

In case of spillage on the heating block, clean the heating block and the light guides underneath the block.

The following items are required:

- Cotton swabs
- Ethanol (70%)
- A clean, non-abrasive, lint-free cloth (e.g., a camera lens cloth).

Procedure:

1. When the instrument is not processing, select "Shutdown" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".
2. When the message "It is now safe to turn off the instrument" appears, switch off the instrument. The power switch is located at the rear of the instrument.
3. Disconnect the instrument from the mains power. There are two mains plugs.
4. Open the instrument lid.
5. Open the plate-holding frame.

Maintenance Procedures


6. Clean each well hole/light guide carefully using cotton swabs lightly moistened with 70% ethanol (see image below).
7. Clean the space between the heating block and the light guide block by carefully inserting a clean, non-abrasive, lint-free cloth lightly moistened with 70% ethanol (see image below).

Caution: Do not use paper tissues.

8. Close the plate-holding frame and the instrument lid and reconnect the instrument to the mains power.



6.3 Maintenance of the PyroMark Q24 Vacuum Workstation

WARNING 	Hazardous chemicals [W5] 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 Material Safety Data Sheets (MSDSs) or OSHA,* ACGIH, [†] or COSHH [‡] documents. For more information, see www.qiagen.com/support/msds.aspx . Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.
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* OSHA: Occupational Safety and Health Administration (United States of America).

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

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

6.3.1 Cleaning the PyroMark Q24 Vacuum Workstation

If the vacuum workstation needs to be cleaned to remove dust and spillage, follow the instructions below.

The following items are required:

- Powder-free gloves
- Nuclease-free Water (cat. no. 129114)
- A mild detergent (if necessary)
- Clean, lint-free cloths.

Procedure:

1. Ensure that no vacuum is applied to the vacuum prep tool, i.e. the vacuum switch is closed (Off), and the vacuum pump is switched off.
2. Disconnect the vacuum pump from the mains power.
3. Clean the worktable and the tool, except for the filter probes, using a clean, lint-free cloth moistened with water or a mild detergent.
Do not touch the tips of the filter probes.
4. Wipe the worktable and the tool, except for the filter probes, dry using a clean, lint-free cloth.
5. Reconnect the vacuum pump to the mains power.

6.3.2 Testing and replacing the filter probes

Function test for filter probes

The function test for the filter probes is described in Section 5.3.1.

Replacing filter probes

Each filter probe can be replaced individually. To ensure proper flow rate through the filter probes, all probes should be replaced after preparation of approximately 100 plates.

Note: Use gloves (powder-free) to avoid contaminating the filter probes.

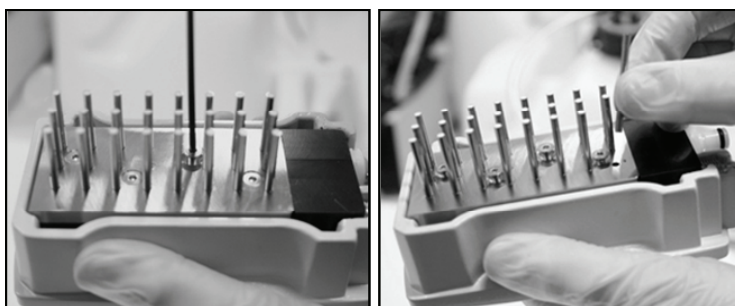
The following items are required:

- Powder-free gloves
- 2-mm Allen key (supplied with the system)
- Nuclease-free Water (cat. no. 129114)
- New filter probes (supplied by QIAGEN).

Procedure:

1. Ensure that no vacuum is applied to the vacuum prep tool, i.e. the vacuum switch is closed (Off), and the vacuum pump is switched off.
2. Disconnect the vacuum pump from the mains power.
3. Remove the tool from the tubing.

4. Loosen the four screws using the 2-mm Allen key supplied with the system.
5. Pull out the old filter probes.
6. Gently insert new filter probes without pressing on the filter tips.
7. Replace and fasten the four screws and reconnect the vacuum pump to the mains power.



6.3.3 Replacing the rubber seal

If the filter probes are loose and/or fall out, there are two possible causes:

- The four screws are not tight enough
- The rubber seal needs to be replaced.

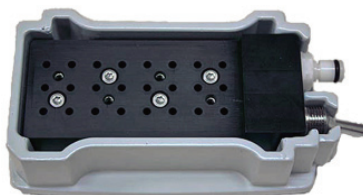
If the rubber seal needs to be replaced, the following items are required:

- Powder-free gloves,
- 2-mm Allen key (supplied with the system)
- New rubber seal (supplied by QIAGEN).

Procedure:

1. Ensure that no vacuum is applied to the vacuum prep tool, i.e. the vacuum switch is closed (Off), and the vacuum pump is switched off.
2. Disconnect the vacuum pump from the mains power.
3. Remove the tool from the tubing.
4. Remove the four screws using the 2-mm Allen key supplied with the system.
5. Gently remove the filter probes. Avoid contaminating the filter probes.

6. Remove the metal plate and replace the rubber seal.



7. Reassemble the tool and reconnect the vacuum pump to the mains power.
8. Check that the filter probes are functioning correctly by performing the function test, as described in Section 5.3.1.

6.3.4 Replacing the tubing

If the tubing is broken or distorted, replace it.

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

The following items are required:

- New tubing (supplied by QIAGEN)
- Beaker.

Procedure:

1. Ensure that no vacuum is applied to the vacuum prep tool, i.e. the vacuum switch is closed (Off), and the vacuum pump is switched off.
2. Disconnect the vacuum pump from the mains power.
3. Remove the broken tubing at one end and empty any liquid waste into an empty beaker.
4. Disconnect the other end of the tubing and discard the tubing and any liquid waste.
5. Cut the new vacuum tubing into three pieces and assemble it. Ensure that the tubing is connected to the pump's vacuum fitting.
6. Reconnect the vacuum pump to the mains power.

6.3.5 Replacing the waste filter

If the waste filter is wet (e.g., if the waste container is full), no vacuum is attained and the filter must be replaced.

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

The following items are required:

- New waste filter
- Beaker.

Note: Two waste filters are supplied with the vacuum workstation. Filters can be ordered at www.millipore.com (Millipore Millex-FG50 Filter Unit, Catalogue Number SLFG05010).

Procedure:

1. Ensure that no vacuum is applied to the vacuum prep tool, i.e. the vacuum switch is closed (Off), and the vacuum pump is switched off.
2. Disconnect the vacuum pump from the mains power.
3. Remove the tubing from the filter fittings and empty any liquid waste into a beaker.
4. Discard the filter.
5. Push the tubing onto the fittings of the new filter.
6. If necessary, empty the waste container.
Note: The cap can be removed without disconnecting the tubing.
7. Reconnect the vacuum pump to the mains power.

Maintenance Procedures

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7 Troubleshooting

If you need to contact QIAGEN Technical Services about an error, note down the steps leading to the error and any information given in any dialog boxes. This will help the QIAGEN Instrument Service Specialist in solving the problem.

When calling QIAGEN Technical Service about errors, please have the following information ready:

- Instrument serial number, type, and version
- Date of last maintenance performed
- Error code (if applicable)
- Time point when the error occurred for the first time
- Frequency of error occurrence (i.e., intermittent or persistent error)
- Photo of error, if possible

Take the following action before contacting QIAGEN Technical Services.

1. Check the run log to assess if the system was working properly during the run.
2. Consult the Troubleshooting sections below.
3. Verify proper installation and operation of your system using PyroMark Control Oligo.

Checking the run log

It is advisable to check the run log to assess if the system was working properly during the run.

1. Open the run file.
2. Select "Run Information" from the "Tools" menu. The Run Information report is opened.
3. Check the run log (at the end of the report) for any problems during the run.
4. If deviations from the preset block temperature, pressure and/or mixer values are noticed several times during a run and for longer time periods or in repeated runs, please contact QIAGEN Technical Services. If requested to send an Environment Data report:

Troubleshooting

- Select "Export Environment Data" from the "Tools" menu
- Select the destination folder from the "Save in" drop-down list
- Enter the filename in the "File name" text box and click "Save".

7.1 Analysis-related errors

Comments and suggestions

Low or missing peaks in the Pyrogram

- | | |
|--|---|
| a) PCR failed due to low DNA quality | Check the PCR samples using a gel technique to confirm there is one strong specific band. If not, rerun PCR with high-quality DNA. |
| b) Poorly optimized PCR | Check the PCR samples using a gel technique to confirm that you have one strong specific band. If not, reoptimize PCR. |
| c) Biotinylation is omitted or not added to the correct PCR primer | Check assay design, see Appendix B, page B-1. |
| d) Biotinylation is of poor quality | Use a recommended primer supplier. Ensure the biotinylated primer is HPLC-purified or similar. |
| e) Insufficient amount of template for immobilization to Sepharose beads | Follow the recommendations for amount of template, see Appendix B, page B-1. |
| f) Not enough enzyme or substrate for all wells | Fill the reagent cartridge according to the Pre Run Information report (open the run setup and select "Pre Run Information" from the "Tools" menu). |

Comments and suggestions	
g) Too much PCR product depletes substrate, leading to missing peaks at the end of the sequence	Use less PCR product.
h) Samples not prepared according to instructions	Prepare samples according to the instructions in Section 5.3.
i) Filter probes not working correctly	Ensure filter probes are working correctly, see Section 5.3.1.
j) Vacuum lost during sample preparation	Ensure that the tubing is connected properly and there is no leakage. The waste filter may be wet and require replacing, see Section 6.3.5.
k) The wells marked in the run setup do not agree with the sample placement on the plate for immobilization or PyroMark Q24 Plate	Check the plate was loaded for immobilization and PyroMark Q24 Plate according to the plate setup.
l) One or several of the nucleotide compartments in the reagent cartridge were not correctly filled	Be sure to add enough reagents (open the run setup and select "Pre Run Information" from the "Tools" menu). Follow the instructions for use supplied with the products.
m) One of the nucleotide needles in the reagent cartridge is blocked or damaged	Clean the reagent cartridge and check that it is working correctly.

Troubleshooting

Comments and suggestions	
n) No enzyme or substrate was added to the well (noted as a missing presequencing signal and missing peaks on the Pyrogram)	Clean the reagent cartridge and check that it is working correctly.
o) Obstructed or damaged reagent cartridge needles	Clean the reagent cartridge and check that it is working correctly. In case of bent needles, discard the reagent cartridge according to federal, state, and local environmental regulations for disposal of laboratory waste.
p) Reagents incorrectly diluted or stored	Be sure to follow the instructions supplied with PyroMark Q24 Gold Reagents.
q) The reagent cartridge is incorrectly inserted	Ensure that the cartridge is inserted correctly
r) The instrument has been started without a plate inserted	Clean the heating block and light guides, see Section 6.2.2.
s) Low signal due to dirty light guides	Clean the heating block and light guides, see Section 6.2.2.

Poor or faulty sequence

a) Sample not prepared according to instructions	Prepare samples according to the instructions in Section 5.3.
b) Low quality in DNA PCR	Check the PCR samples using a gel technique to confirm that you have one strong specific band. If not, reoptimize PCR.

Comments and suggestions	
c) Incorrect sequence to analyze	Check typing and reference sample.
d) Background of contaminating sequence from PCR	Check the PCR samples using a gel technique to confirm that you have one strong specific band. If not, re-optimize PCR.
e) Sequence signals from self-annealed sequencing primer and/or biotinylated PCR primer	Follow the recommendations in Appendix B, (page B-1) the first time an assay is run.
f) Unspecific annealing of sequencing primer	Follow the recommendations in Appendix B, (page B-1) the first time an assay is run. Redesign the sequencing primer.
g) Sequence signal from self-annealed template	Follow the recommendations in Appendix B, (page B-1) the first time an assay is run. Redesign the nonbiotinylated primer or add NNN at the 5' end.
h) Contaminated sample has led to unusually high consumption of substrate (noted as a high presequencing signal)	Prepare samples according to the instructions in Section 5.3. Change buffers and do not use any other buffers than those supplied by QIAGEN. Check if any peaks have been generated using the zoom in function (select a stretch of Pyrogram with the left mouse button).
i) Reagents incorrectly diluted or stored	Be sure to follow the instructions supplied with PyroMark Q24 Gold Reagents. Do not store nucleotides at –20°C. Include an empty well (Annealing Buffer only) in the run setup to check whether background peaks are coming from nucleotides.

Troubleshooting

Comments and suggestions

- | | | |
|----|---|--|
| i) | Crosstalk (light from one well appears in the neighboring well) | Avoid placing assays with high signals close to assays with low signals. |
| k) | Dispensation error | Replace the reagent cartridge. If the problem remains, contact QIAGEN Technical Services. |
| l) | Unknown SNP in sample | Insert the SNP in the sequence to analyze and regenerate the dispensation order. Rerun the sample with the new dispensation order. |
| m) | dUTP used in the PCR reaction | Replace dUTP with dTTP since the A nucleotide used in Pyrosequencing reactions binds less stringently to dUTP. |
| n) | Plus shift | Change the dispensation order. |
| o) | Minus shift | Ensure that homopolymers are followed by an extra dispensation. |

7.2 Analysis software-related errors

For errors related to the analysis software, see the Troubleshooting section of the *PyroMark Q24 Analysis Software User Guide*.

7.3 Instrument-related errors

Comments and suggestions

Error messages

- | | | |
|----|--|--|
| a) | Too many unsaved runs in the instrument. Please go to folder "Unsaved Runs" and save them to USB stick | Transferred unsaved runs to a USB stick. |
|----|--|--|

Comments and suggestions

- | | |
|--|---|
| b) The required value was not reached.
The run will be stopped | Restart the run. If the room temperature is high and a temperature problem remains: <ul style="list-style-type: none"> ■ Ensure the cooling device is receiving power; a light indicator at the rear is lit. If not, check your connections. ■ Check the coolant level. |
| c) "Run name" is invalid. | Ensure that the run file is created in PyroMark Q24 Software. |
| d) Could not copy "file" to USB stick | Try another USB stick. We recommend using USB sticks supplied by QIAGEN. |
| e) Failed to connect to the mixer, please restart the instrument | Restart the instrument. If the problem remains, contact QIAGEN Technical Services. |
| f) Failed to connect to the hardware, please restart the instrument | Restart the instrument. If the problem remains, contact QIAGEN Technical Services. |
| g) Connection to the hardware is lost, please restart the instrument | Restart the instrument. If the problem remains, contact QIAGEN Technical Services. |
| h) No valid upgrade folders/files found on USB stick | Ensure your upgrade installation files are located in a folder called Upgrade at the root of the stick. |
| i) "Unit" failed | Contact QIAGEN Technical Services. |
| j) Automatic recovery of "Run name" failed | Contact QIAGEN Technical Services. |

Troubleshooting

Comments and suggestions

- | | | |
|----|--|------------------------------------|
| k) | Failed to clear temporary file storage | Contact QIAGEN Technical Services. |
| l) | Failed to create log files | Contact QIAGEN Technical Services. |
| m) | Failed to create run file "filename" | Contact QIAGEN Technical Services. |
| n) | Failed to extract damaged runs | Contact QIAGEN Technical Services. |
| o) | Failed to set time and date | Contact QIAGEN Technical Services. |
| p) | Internal memory full | Contact QIAGEN Technical Services. |
| q) | Upgrade failed | Contact QIAGEN Technical Services. |

Other problems

- | | | |
|----|---|--|
| a) | The instrument is making unexpected noise when starting | Check that the cartridge is inserted correctly. |
| b) | No contact with the USB stick | The used USB stick is damaged or not compatible with the system. We recommend using USB sticks supplied by QIAGEN. |
| c) | USB stick can not be inserted | Broken USB contact, contact QIAGEN Technical Services. |

7.4 Vacuum Workstation-related errors

	Comments and suggestions
a) No vacuum is received	<p>Turn off the vacuum pump and open the cap to the waste container to release any pressure. Close the cap and start the pump again. Empty the waste container if full.</p> <p>Ensure that the tubing is connected correctly and that there is no leakage.</p> <p>The waste filter may need to be replaced, see Section 6.3.5.</p>
b) Liquid left in some wells in the immobilization plate	<p>Replace the corresponding filter probe, see Section 6.3.2.</p>
c) White remains (Sephacrose beads) in the immobilization plate	<p>Do not leave the plate longer than 1 minute after mixing is finished. If necessary, mix for 1 minute extra.</p>

7.5 Verification of correct installation and operation

PyroMark Control Oligo is sold together with the PyroMark Q24 System to verify proper installation and operation of the system. The Control Oligo consists of one wobbled base (measured as %C), single bases of all four nucleotides, and homopolymers of two and three bases. For information on how to use the Control Oligo, see the instructions supplied with the product.

Troubleshooting

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8 Glossary

Term	Description
Biotin	A molecule that can bind very strongly to streptavidin. PCR primers can be biotinylated to enable the resulting PCR product to bind to streptavidin-coated Sepharose beads.
Bisulfite	HSO_3^- is referred to as bisulfite (or hydrogen sulfite). In the bisulfite reaction, DNA is treated with sodium bisulfite to convert cytosine residues to uracil, under conditions whereby methylated cytosines remain non-reactive.
Bisulfite treatment control	Pyrosequencing assays can contain an internal control to assess successful bisulfite treatment. C bases that are not followed by G in the sequence are normally not methylated, and should therefore be fully converted to T after bisulfite treatment and PCR. As a result of successful bisulfite treatment, all templates should show only Ts and no Cs in these positions. For reverse assays, all templates should show only As and no Gs in these positions.
Cyclic dispensation order	A repetitive dispensation order for nucleotide dispensation. Normally used in Pyrosequencing technology for sequencing unknown DNA-sequences. For example, "CTGA" or "TCGA" can be used and repeated for the desired number of times.
Directed dispensation order	Non-cyclic order of dispensation that follows the known sequence. It can be used in Pyrosequencing technology when you know the sequence to be analyzed. For example, the sequence "TCCAGAA" can be analyzed with the dispensation order "TCAGA".
Dispensation order	Defines the nucleotides and the order in which they should be dispensed in Pyrosequencing runs.
Drop off	A continual decrease in signal height normally seen in the Pyrogram.

Glossary

Term	Description																
Enzyme	A protein (or RNA) working as a catalyst, to enhance the speed of a biochemical reaction without altering it. In Pyrosequencing technology, a mixture of Klenow polymerase, sulfurylase, luciferase, and apyrase is used in the sequencing reaction.																
Histogram	The theoretical representation of the expected Pyrosequencing peak pattern.																
Homopolymer	A stretch of identical bases in DNA. In Pyrosequencing technology, a stretch of more than two identical bases is regarded as a homopolymer.																
Instrument methods	A method that describes physical settings for the instrument, such as mixer frequency, block temperature, and pulse time settings.																
IUPAC	<p>International Union of Pure and Applied Chemistry. An organization providing recommendations on organic and biochemical nomenclature, symbols, terminology, etc.</p> <p>IUPAC codes</p> <table><tr><td>A = Adenine</td><td>K = T or G</td></tr><tr><td>C = Cytosine</td><td>W = T or A</td></tr><tr><td>G = Guanine</td><td>S = C or G</td></tr><tr><td>T = Thymine</td><td>B = C, T, or G (not A)</td></tr><tr><td>U = Uracil</td><td>D = A, T, or G (not C)</td></tr><tr><td>R = Purine (A or G)</td><td>H = A, T, or C (not G)</td></tr><tr><td>Y = Pyrimidine (C or T)</td><td>V = A, C, or G (not T)</td></tr><tr><td>M = C or A</td><td>N = Any base (A, C, G, or T)</td></tr></table>	A = Adenine	K = T or G	C = Cytosine	W = T or A	G = Guanine	S = C or G	T = Thymine	B = C, T, or G (not A)	U = Uracil	D = A, T, or G (not C)	R = Purine (A or G)	H = A, T, or C (not G)	Y = Pyrimidine (C or T)	V = A, C, or G (not T)	M = C or A	N = Any base (A, C, G, or T)
A = Adenine	K = T or G																
C = Cytosine	W = T or A																
G = Guanine	S = C or G																
T = Thymine	B = C, T, or G (not A)																
U = Uracil	D = A, T, or G (not C)																
R = Purine (A or G)	H = A, T, or C (not G)																
Y = Pyrimidine (C or T)	V = A, C, or G (not T)																
M = C or A	N = Any base (A, C, G, or T)																
Out of phase	When one of the alleles is sequenced ahead of the other.																

Term	Description
Polymorphism	Genetic variations, broadly encompassing any of the many types of variations in DNA sequences that are found within a given population.
Presequencing signal	As the substrate is dispensed into a well (indicated by an "S"), contaminants (such as ATP or PPi) will be converted to light. Too high a substrate peak indicates that high levels of a contaminant might be present in, for example, buffers.
Pyrogram	The graph resulting from a sequencing reaction performed using Pyrosequencing technology. Each incorporated nucleotide is shown as a peak in Pyrogram.
Reference peak	Nonvariable peaks, i.e. peaks that are not a part of an SNP or CpG site, are referred to as "reference peaks". Reference peaks are used in the analysis both as references when calculating the single peak height, and as internal controls when assessing the quality.
RLU	Relative Light Unit (entity used in Pyrosequencing to define peak heights in Pyrogram).
Sepharose beads	Streptavidin-coated beads can be used for preparation of biotinylated PCR products.
Sequence to analyze	A short part of a DNA sequence (in your sample), starting directly after the sequencing primer, which contains one or several variable positions to be analyzed using Pyrosequencing instrument platforms.
Sequencing primer	The sequencing primer is annealed to the template during the sample preparation. The 3'-end of the sequencing primer serves as the starting point for the extension by the polymerase.

Glossary

Term	Description
Shift	<p>Positive shift: A small proportion of the template sequences that incorporates more than one type of nucleotide at a time (if, for example, there are residues left from the dispensation before) and will be sequenced ahead of the rest of template sequences.</p> <p>Negative shift: A small proportion of the template sequences that fails to incorporate a nucleotide will be sequenced subsequent to the rest of template.</p>
Signal-to-noise ratio	The ratio of the signal height and the noise height. An indication of the clarity of the data. The higher the ratio, the better the data.
Single nucleotide polymorphism (SNP)	SNPs involve the change of one DNA base to another. SNPs and point mutations are structurally identical, differing only in their frequency. Variations that occur in 1% or less of a population are considered point mutations, while those occurring in more than 1% are SNPs.
Streptavidin	A protein that can bind very strongly to biotin.
Substrate	A molecule acted upon by an enzyme. Pyrosequencing technology uses a mixture of the substrates Adenosine 5' phosphosulfate (APS) and Luciferin in the sequencing reaction.
Theoretical outcome	The possible genotypes for a specific polymorphic position are calculated on the basis of the sequence to analyze and the dispensation order.
Variable position	A region in the dispensation order where the sequence varies at one or more variable bases. In Pyrosequencing software, the variable positions are highlighted with a blue-gray background color in the histogram and Pyrogram.

Appendix A

Technical data

QIAGEN reserves the right to change specifications at any time.

Environmental conditions

Operating conditions: PyroMark Q24

Power 100–240 VAC, 50–60 Hz, 160 VA

Overvoltage category II

Air temperature 15–32°C (59–90°F)

Relative humidity 20–90% (noncondensing)

Altitude Up to 2000 m (6500 ft.)

Place of operation For indoor use only
Draft-free location, not close to window. Keep instrument out of direct sunlight

Pollution level 2

Environmental class 3K2 (IEC 60721-3-3)

Transportation conditions

Air temperature –25°C to 60°C (–13°F to 140°F)

Relative humidity Max. 75% (noncondensing)

Appendix A

Storage conditions

Air temperature 10°C to 40°C (50°F to 104°F)

Relative humidity Max. 75% (noncondensing)

Mechanical data and hardware features

Dimensions (closed) Width: 390 mm (15.35 in.)
Height: 420 mm (16.54 in.)
Depth: 525 mm (20.67 in.)

Clearance space Width: 700 mm (27.56 in.)
Height: 700 mm (27.56 in.)
Depth: 600 mm (23.62 in.)

Mass 28 kg (61.74 lb.)

Capacity Up to 24 samples per run

Chemical resistance pH 4 to pH 9, common detergents, 0.5 M sodium hydroxide, 70% ethanol

Operating conditions: PyroMark Q24 Vacuum Workstation

Power 100 VAC, 50/60 Hz, 1.7/1.4 A, or
115 VAC, 60 Hz, 1.5 A, or
230 VAC, 50 Hz, 0.6 A
Power consumption: Maximum 25 Watt

Overvoltage category II

Air temperature 15–32°C (59–90°F)
We recommend that the instrument is run at room temperature (15–25°C) for optimal performance.

Relative humidity 20–90%

Altitude Up to 2000 m (6500 ft.)

Place of operation	For indoor use only Normal laboratory conditions; use adequate ventilation
Pollution level	2
Environmental class	3K2 (IEC 60721-3-3)

Transportation conditions

Air temperature -25°C to 60°C (-13°F to 140°F)

Relative humidity Max. 75% (noncondensing)

Storage conditions

Air temperature 10°C to 40°C (50°F to 104°F)

Relative humidity Max. 75% (noncondensing)

Mechanical data and hardware features

Dimensions (worktable)	Width: 295 mm (11.61 in.) Height: 68 mm (2.68 in.) Depth: 353 mm (13.90 in.)
Clearance space	Width: 350 mm (13.78 in.) (or 700 mm [27.56 in.]) Height: 400 mm (15.74 in.) Depth: 700 mm (27.56 in.) (or 350 mm [13.78])
Mass	11 kg (24.26 lb.)
Capacity	1–24 samples
Process time	Less than 5 minutes for up to 24 samples in parallel
Chemical resistance	pH 4 to pH 9, common detergents, 0.5 M sodium hydroxide, 70% ethanol

Appendix A

PyroMark Q24 Software

Operating system	Microsoft Windows XP or Windows Vista, English versions
Processor	Intel Pentium IV, 3 GHz (or higher)
RAM	1 GB
Free hard disk space	100 MB
Graphics card	Supporting the resolution of the monitor
Monitor	1280 x 1024 pixels
Pointer device	Mouse or similar
Interfaces	USB port and CD-ROM

Waste Electrical and Electronic Equipment (WEEE)

This section provides information about disposal of waste electrical and electronic equipment by users in the European Union.

The European Directive 2002/96/EC on WEEE requires proper disposal of electrical and electronic equipment when it reaches its end of life. The crossed-out wheeled bin symbol (see below) indicates that this product must not be disposed of with other waste; it must be taken to an approved treatment facility or to a designated collection point for recycling, according to local legislation. The separate collection and recycling of waste electronic equipment at the time of disposal helps to conserve natural resources and ensures that the product is recycled in a manner that protects human health and the environment.



QIAGEN accepts its responsibility in accordance with the specific WEEE recycling requirements and, where a replacement product is being supplied by QIAGEN, provides free recycling of its WEEE-marked electronic equipment in Europe. If a replacement product is not being purchased from QIAGEN, recycling can be provided upon request at additional cost. To recycle electronic equipment, contact your local QIAGEN sales office for the required return form. Once the form is submitted, you will be contacted by QIAGEN either to request follow-up information for scheduling collection of the electronic waste or to provide you with an individual quote.

Appendix A

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Appendix B

Assay design and validation

Summary

PCR

- Use Pyrosequencing Assay Design Software for primer design.
- Ensure oligonucleotides are of a high quality (including HPLC purified, biotinylated primer).
- For CpG assays, use fresh reagents or commercial kits for bisulfite treatment.
- Use hot-start DNA polymerase in PCR (QIAGEN recommends HotStarTaq® DNA Polymerase, cat. no. 203203 or HotStarTaq Master Mix Kit, cat. no. 203443)
- Ensure there is sufficient DNA in PCR.
- Aim for PCR products that give a single strong band on an agarose gel with no excess of primers.
- Test for equal amplification of both alleles.

Pyrosequencing analysis

When creating an assay:

- Include at least five reference peaks.
- Include blank dispensations – they are automatically generated by the software.
- Make suitable modifications when the red warning icon appears (🚫)
- Avoid homopolymers in variable positions, if possible
- Use C, G, or T in variable positions, if possible.

Controls in first run of new assay:

- PCR without DNA
- Sequencing primer alone
- Template without sequencing primer.

Analysis

- Ensure you use appropriate analysis parameters.

For samples and positive controls, aim for:

- Sufficient signal intensities. Aim for a single peak height of at least 20 RLU
- No background in blank dispensations
- No background in variable positions
- Expected reference sequence pattern
- All positions with quality assessment "Passed".

Detailed description

Assay design

Primers should be designed with Pyrosequencing Assay Design Software (ADSW). The program automatically generates primer sets that include both PCR and sequencing primers. Each primer set is given a quality score based on several parameters that are specific for Pyrosequencing analysis.

PCR primers

PCR primers should be 18–24 bases in length, with annealing temperatures that are similar and typically in the range 60–70°C (nearest neighbor method).

One of the primers must be biotin-labeled to enable immobilization to streptavidin-coated Sepharose beads during the preparation of a single-stranded DNA template. The orientation of the assay can either be forward or reverse. The primer that needs to be biotinylated is indicated.

The primers should not form strong hairpin loops or dimers with themselves or with the other primers. The biotinylated primer should be carefully checked for hairpin loops and duplexes with the sequencing primer, as excess biotinylated primer might cause background in Pyrosequencing assays. If possible, avoid placing primers over polymorphic positions.

The biotinylated primer should be purified by HPLC or an equivalent procedure since free biotin will compete with the biotinylated PCR product for binding sites on streptavidin-coated Sepharose beads.

Amplicon length

The optimal amplicon length is between 80–200 bp, although products up to 500 bp may work well.

Sequencing primer

The sequencing primer should be 15–20 bases long and have an annealing temperature in the range 45–55°C. The sequencing primer should differ from the PCR primer by at least one additional, specific base at the 3' end.

Specific considerations for CpG assays

In CpG assays, the optimal length of the primers is slightly increased to function on bisulfite-treated DNA, which has a high proportion of A and T. PCR primers should usually be 22–30 bases and sequencing primers should be 18–23 bases long.

Since bisulfite treatment gives low quality DNA, the amplicons for CpG assays should be shorter than 200 bp.

If sequences have densely packed CpG sites, the design may require manual intervention. However, the penalties and scores given by the software are still very informative.

Manual operations for CpG assay design can include:

- Looking for the region of most interest and selecting it as the target
- Placing PCR primers without covering more than one CpG site
- Ensuring the CpG site is in the 5' part of the primer, if a primer needs to be located over a CpG site
- Looking for warnings and checking for analysis parameters that can be improved (T_m for primers, mispriming, duplexes between biotinylated primer and sequencing primer, amplicon length, etc.)
- Improving the final score given by the Pyrosequencing Assay Design Software by changing the length of the primers and by moving them back and forth.

PCR setup

PCR reactions of 25 μ l are set up using a hot start polymerase. Ensure that you follow the instructions provided with the PCR reagents.

One of the primers must be labeled with biotin for immobilization to Streptavidin Sepharose High Performance beads (GE Healthcare, cat. no. 17-5113-01) during the sample preparation step.

PCR mixture

- PCR buffer
- 1.5–3 mM MgCl₂
- 5 pmol forward PCR primer (200 nM)
- 5 pmol reverse PCR primer (200 nM)
- 0.2 mM of each dNTP (dNTP Set, PCR grade [4 x 100 μ l], cat. no. 201912)
- Units DNA polymerase (HotStarTaq [250 U], cat. no. 203203; HotStarTaq Master Mix Kit [250 U], cat. no. 203443)
- 10 ng genomic DNA
- Nuclease-free water (cat. no. 129114, filtered through 0.22 μ m filter) to 25 μ l.

The annealing temperature is usually in the range 55–60°C. Run the PCR at the optimal annealing temperature for 45 cycles. Using fewer cycles may give insufficient yield and cause background problems in Pyrosequencing reactions due to excess, unused biotinylated primer.

The PCR product should give one strong band with minimal excess of primers when analyzed on an agarose gel.

PCR optimization

The quality of DNA for PCR optimization should be as high as possible. We recommend using the EpiTect® Bisulfite Kit for bisulfite treatment. The unique DNA protection mechanism included in the DNA Protect Buffer prevents DNA fragmentation, which is usually associated with bisulfite

treatment if using home brew methods, for example. The prevention of fragmentation enables reliable amplification.

Evaluate PCR conditions carefully. The optimal ranges for annealing temperature and MgCl_2 concentration may be very narrow, especially for a CpG assay. We recommend testing at least two MgCl_2 concentrations, e.g., 1.5 mM and 3 mM. The annealing temperature gradient should span 15°C for both magnesium concentrations, at intervals of approximately 3°C.

For all PCR optimization tests, analyze 1/10 of the PCR on an agarose gel and aim for one strong specific band with minimal excess of primers.

Equal amplification of both alleles

Reliable results in quantification assays depend on equal amplification of both alleles and this must be carefully tested.

To ensure equal amplification in a CpG assay, unmethylated DNA can be mixed with increasing proportions of completely methylated DNA. We recommend using EpiTect Control DNAs, which provide bisulfite-treated completely methylated and unmethylated DNA in ready-to-use solutions. Regression analysis of the frequency of one allele measured in PyroMark Q24 as a function of the input (expected) allele, should give an R^2 value greater than 0.9.

For an AQ assay, the allelic variants being part of the variable position can be mixed at different ratios similar to the procedure for a CpG assay. If the variable position in an AQ assay is an SNP, the easiest way to test for equal amplification is to compare the peak heights from a heterozygote. If the SNP is represented by single base incorporations, e.g., AAC/TGG, the two alleles (C and T peaks) should give peaks of equal height.

Sample preparation

Use 10–20 μl of a 25 μl PCR for immobilization to Streptavidin Sepharose High Performance (GE Healthcare) according to instructions in the manual. This will be

equivalent to 1–4 picomoles of PCR product providing the recommended PCR protocol has been used to develop a well-optimized assay.

Assay setup

The sequence to analyze should contain a sufficient number of bases to generate at least five nonvariable reference peaks. If the sequencing primer is placed adjacent to the position to analyze, include part of the sequence following the variable position in the “Sequence to Analyze” text box. Ensure that the last base in the text box is not part of the variable position.

Blank dispensations are automatically generated by the software and serve as built-in quality controls for the assay. Never exclude the blank dispensations as they function as excellent indicators of unspecific nucleotide incorporation. When manually generating a dispensation order, include an appropriate number of blank dispensations. If possible, start the dispensation order with one blank dispensation and have at least the same number of blank dispensations as the number of variable positions.

Pay attention to tips and warnings indicated by the red icon (🔴).

Variable positions

If possible, variable positions should not include homopolymers (a stretch of two or more nucleotides of the same kind). If the variable position is preceded by a homopolymeric stretch, place the sequencing primer adjacent to the position to analyze. If the variable position is followed by a homopolymeric stretch, change the orientation of the assay.

C, G, and T are the preferred bases in variable positions. “A peaks” are slightly higher than the other three base peaks due to the chemistry of the modified A-nucleotide (dATP α S). In exceptional cases the heights of the A peaks may vary between assays. This can be compensated by changing the A peak reduction factor at the “Analysis Parameters” tab.

CpG assays

When creating a CpG assay, the software indicates possible dispensations that can be controls for completion of bisulfite treatment. The preferable controls are those dispensations that are located in the beginning of the sequence and/or represent single base incorporations.

Note: In the sequence before bisulfite treatment, check whether the suggested bisulfite controls are Cs converted to Ts (read as Gs and As in a reverse assay) or not.

Validation of a new assay

Controls

Use a reference DNA sample when testing a new assay and ensure that appropriate analysis parameters in PyroMark Q24 Software are used. Interactions between primers or loops formed on single-stranded DNA can serve as priming sites for base incorporation by DNA polymerase. The following controls should be included when an assay is analyzed for the first time.

- A PCR without template DNA. This will show if the primers interact to give a background signal in Pyrosequencing reactions.
- A PCR with template DNA but with no sequencing primer. This will show if the template can loop back on itself and give a background signal in Pyrosequencing reactions.
- A sequencing primer without any PCR product. This will show if the sequencing primer can form duplexes or hairpins and give background signal in Pyrosequencing reactions.

Pyrogram traces from these controls should not show any peak after any nucleotide addition.

Quality assessment

The user will be warned if something in the assay may reduce the quality of the result given by the analysis software. The default settings for the analysis parameters are chosen to

indicate the seriousness of the problem by giving a “Check” or “Failed” quality message. The ultimate goal for a well-optimized assay is that all positions have the quality assessment “Passed” when using default or more stringent analysis parameters. Such results will be shown as an entirely blue quality bar in the well after analysis. Results of lower quality are indicated as “Check” (yellow) or “Failed” (red) together with error messages.

Analysis results

The analysis results for an assay are based on the sequence context as well as the results in the analyzed position. Deviations from this built-in control are shown as warnings in the Well Information area.

Deviations in reference sequence pattern

Reliable results depend on correct peaks with sufficient signal intensities. The default setting for required single peak height is 20 RLU. Blank dispensations are automatically included in the assay design by the software and these serve as controls for unspecific incorporation. If peaks of a certain height are generated at these positions, the warning Failed/Uncertain reference sequence pattern at dispensation: N is shown in the Well Information area.

The warning “Failed/Uncertain reference sequence pattern at dispensation: N” also appears if incorporation at a certain dispensation results in a peak that deviates from the expected height. If several peaks deviate from the expected height, the warning “Failed/Uncertain surrounding reference sequence pattern” appears. Both warnings, with the second being much more severe, are indications that the assay needs further optimization in terms of design or PCR conditions.

Deviation in sum or pattern in variable position

Unexpected peak height in the variable position results in the warning “Failed/Uncertain due to high sum deviation in variable position”, “Failed/Uncertain due to high pattern

deviation in variable position", or "Failed/Uncertain due to high peak height deviation at dispensation: N". For AQ assays, both homozygotes should be analyzed and it is important that there is no background incorporation from the other homozygote. For a CpG assay, both completely unmethylated and fully methylated samples should be analyzed. However, it may be difficult to obtain both 0% and 100% methylated material. If several sites are included in an assay and one of these warnings occurs, provided that it appears for just one position and no other warnings appear, this position can simply be excluded from the analysis.

Appendix B

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Appendix C

PyroMark Q24 accessories

Product	Contents	Cat. no.
PyroMark Q24	Sequence based detection platform for Pyrosequencing of 24 samples in parallel Instrument, for laboratory use only	9001514
PyroMark Q24 Vacuum Workstation	Vacuum Workstation (220 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001518
PyroMark Q24 Vacuum Workstation	Vacuum Workstation (110 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001516
PyroMark Q24 Vacuum Workstation	Vacuum Workstation (100 V) for preparing 24 samples in parallel, from PCR product to single-stranded template	9001519
PyroMark Q24 Software	Analysis software with two modes (CpG and AQ allele quantification), for laboratory use only	9019062
PyroMark Q24 Vacuum Prep Tool	Vacuum Prep Tool	9019064
Reagents		
PyroMark Gold Q24 reagents (5 x 24)	Enzymes (DNA polymerase, ATP sulfurylase, luciferase, and apyrase), substrates (adenosine 5'phosphosulfate (APS), and luciferin) and nucleotide (one vial each of C, A, T, and G nucleotides)	970802
PyroMark Control Oligo	For installation check of system and troubleshooting	979203
PyroMark Validation Oligo	For performance confirmation of system	979204

Appendix C

Product	Contents	Cat. no.
Buffers		
PyroMark Binding Buffer (200 ml)	For binding of biotinylated PCR product to Sepharose beads	979006
PyroMark Denaturation Solution (500 ml)	For denaturation of double-stranded PCR product into single-stranded template DNA	979007
PyroMark Wash Buffer, concentrate (200 ml)	For washing of single-stranded DNA	979008
PyroMark Annealing Buffer (250 ml)	For annealing sequencing primer to single-stranded PCR product and for Pyrosequencing reaction	979009
Accessories		
PyroMark Q24 Cartridge (3)	Cartridges for dispensing nucleotides	979202
PyroMark Q24 Plate (100)	24-well plate, for pipetting sequencing reactions	979201
PyroMark Q24 plate holder	Holder for 24 well plate	979205
EpiTect Bisulfite Kits — for complete bisulfite conversion and cleanup of DNA for methylation analysis		
EpiTect Bisulfite Kit (48)	48 EpiTect Bisulfite Spin Columns, Reaction Mix, DNA Protect Buffer, Carrier RNA, Buffers	59104

Product	Contents	Cat. no.
EpiTect Control DNA — for evaluation of PCR primers used for methylation analysis		
EpiTect Control DNA, methylated (100)	Methylated and bisulfite converted human control DNA	59655
EpiTect Control DNA, unmethylated (100)	Unmethylated and bisulfite converted human control DNA	59665

Appendix C

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Appendix D

Liability clause

QIAGEN shall be released from all obligations under its warranty in the event repairs or modifications are made by persons other than its own personnel, except in cases where the Company has given its written consent to perform such repairs or modifications.

All materials replaced under this warranty will be warranted only for the duration of the original warranty period, and in no case beyond the original expiration date of original warranty unless authorized in writing by an officer of the Company. Read-out devices, interfacing devices and associated software will be warranted only for the period offered by the original manufacturer of these products. Representations and warranties made by any person, including representatives of QIAGEN, which are inconsistent or in conflict with the conditions in this warranty shall not be binding upon the Company unless produced in writing and approved by an officer of QIAGEN.

Appendix D

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