Version 1 **January 2016**

PyroMark® Q24 MDx User Manual

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MAT 1063078EN

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QIAGEN®

Sample & Assay Technologies

Contents

1 Safety Information

Before using the PyroMark Q24 MDx, 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 system and to maintain the system in a safe condition.

The following types of safety information appear throughout this manual.

WARNING 	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.

CAUTION 	The term CAUTION is used to inform you about situations that could result in **damage to the system** or other equipment. Details about these circumstances are given in a box like this one.

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

WARNING 	**Risk of personal injury and material damage** Improper use of the PyroMark Q24 MDx may cause personal injuries or damage to the system. PyroMark Q24 MDx must only be operated by qualified personnel who have been appropriately trained. Servicing of the PyroMark Q24 MDx must only be performed by QIAGEN Field Service Specialists.

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** The PyroMark Q24 MDx Instrument is too heavy to be lifted by one person. To avoid personal injury or damage to the instrument, do not lift the instrument alone.

WARNING	**Risk of personal injury and material damage** Do not attempt to move the PyroMark Q24 MDx during operation.

1.2 Electrical safety

Disconnect the line power cords from the power outlets before servicing.

WARNING	**Electrical hazard** 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 equipment** When the equipment is connected to line power, terminals may be live, and opening covers or removing parts is likely to expose live parts.

To ensure satisfactory and safe operation of the PyroMark Q24 MDx, follow the advice below:

- The instrument's line power cords must be connected to line power outlets that have protective conductors (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 system.

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).

WARNING	**Biological materials**
	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 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. 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.

* 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**
	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 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.

* 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 MDx Instrument 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**
	To avoid contact with moving parts during operation of the PyroMark Q24 MDx Instrument, 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 MDx Instrument, contact QIAGEN Technical Services immediately.

WARNING 	**Sharp needles** Do not touch the sharp needles at the bottom of the reagent cartridge. Handle the needles with care. Small particles and fibers may obstruct the needles.

CAUTION 	**Light guide maintenance** Use lint free tissues to clean the space between the heating block and the light guide block inside the instrument. Do not use paper tissues.

1.6 Heat hazard

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

CAUTION 	**Risk of overheating** To ensure proper ventilation, maintain a minimum clearance of 10 cm (3.94 in.) at the sides and rear of the PyroMark Q24 MDx Instrument. Slits and openings that ensure the ventilation of the PyroMark Q24 MDx Instrument must not be covered.

1.7 Consumables

CAUTION 	**Unsupported consumables** Do not connect or use any consumables, accessories, or external equipment other than that specified.

1.8 Symbols on the PyroMark Q24 MDx and PyroMark Q24 products

Symbol	Location	Language	Description
	Type plate on the back of the instrument	EN	CE mark
	Type plate on the back of the instrument	EN	CSA listing mark for Canada and the USA
	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	RCM 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	Waste Electrical and Electronic Equipment (WEEE)
	Type plate on the back of the instrument	EN	In vitro diagnostic medical device

Symbol	Location	Language	Description
LOT	Reagents, solutions, cartridge, plate	EN	Identification of production batch/lot
Σ <N>	Reagents, solutions	EN	Contains reagents for <N> tests
REF	Reagents, solutions, cartridge, plate	EN	Catalog number
MAT	Reagents, solutions, cartridge, plate	EN	Material number
COMP	Reagents, solutions, cartridge, plate	EN	Component
CONT	Reagents, solutions, cartridge, plate	EN	Contains
NUM	Reagents, solutions, cartridge, plate	EN	Number
	Reagents, solutions, cartridge, plate	EN	Use-by date
	Reagents, solutions, cartridge, plate	EN	Temperature limitation

Symbol	Location	Language	Description
	Instrument, VPW, reagents, cartridge	EN	Read the manual
	Type plate on the back of the instrument and all other products	EN	Legal manufacturer
	Cartridge	EN	Keep away from (sun)light
METHOD	Cartridge	EN	Method number
INSTRUM-PARAM	Cartridge	EN	Instrument parameters
	Inside the instrument	EN	Warning, consult user manual

2 Introduction

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

Before using the PyroMark Q24 MDx, it is essential that you read this user manual carefully and pay particular attention to the safety information (see Section 1). The instructions and safety information in the user manual must be followed to ensure safe operation of the system and to maintain the system in a safe condition.

2.1 About this user manual

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

1. Safety Information
2. Introduction
3. General Description
4. Installation Procedures
5. Operating Procedures
6. Maintenance Procedures
7. Troubleshooting
8. Glossary

   Appendices

The appendices include the following:

- Technical data
- Environmental conditions
- WEEE recycling requirements
- Assay design and validation
- 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 Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the PyroMark Q24 MDx or QIAGEN products in general, please do not hesitate to contact us.

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

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

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 MDx User Manual*, version 1.0, revision R4.

2.3 Intended use of PyroMark Q24 MDx

PyroMark Q24 MDx is a system for detecting changes in specified variable positions in DNA that may have clinical relevance.

The PyroMark Q24 MDx is intended for in vitro diagnostic use in Europe.

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

The PyroMark Q24 MDx 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 MDx System.

2.3.1 Requirements for PyroMark Q24 MDx users

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

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
Assay design and validation	Scientist or equivalent	Appropriately trained and experienced personnel familiar with molecular biological techniques
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 MDx uses proven real-time sequence-based Pyrosequencing® technology for sequence-based detection and quantification in genetic analysis and epigenetic methylation studies. The system can analyze up to 24 samples simultaneously. An easy-to-use protocol is used to prepare samples.

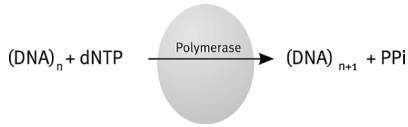
3.1 PyroMark Q24 MDx definitions

- PyroMark Q24 MDx: Instrument, software, and installation
- PyroMark Q24 MDx Instrument: Instrument only
- PyroMark Q24 MDx Software: Software only
- PyroMark Q24 MDx Vacuum Workstation: Vacuum Workstation only
- PyroMark Q24 MDx System: All of the above, plus any PyroMark Kits

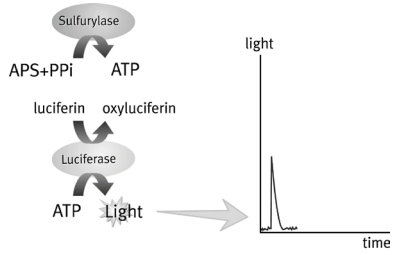
3.2 Pyrosequencing principle

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.



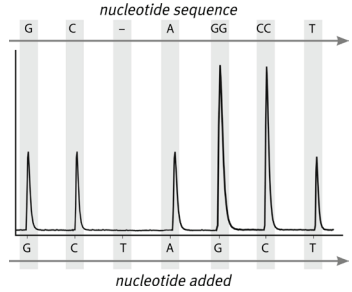
5. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate.
6. This drives the conversion of luciferin to oxyluciferin by luciferase, generating visible light in amounts proportional to the amount of ATP. Light is detected using charged coupled devices (CCDs)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



nucleotide incorporation generates light seen as a peak in Pyrogram

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 (dATPαS) 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 peak in the Pyrogram.



3.3 PyroMark Q24 MDx principle

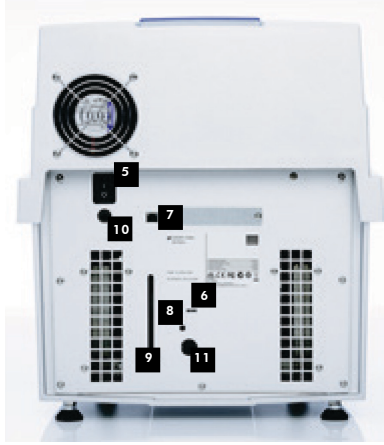
The PyroMark Q24 MDx performs DNA sequencing using Pyrosequencing technology.

1. The PyroMark Q24 Plate containing the samples is placed on the heating block inside the instrument and the PyroMark Q24 Cartridge is filled with PyroMark Gold Q24 Reagents and placed in the dispensing unit.
2. The USB stick containing the run file created using PyroMark Q24 MDx Software is inserted into the USB port at the front of the instrument. The run is then started by the user.
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's priming well (the rectangular well) 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 priming well before being dispensed into the wells. Nucleotides are added in a predefined order 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 are stored on the instrument.
9. After the run, data are automatically transferred to the USB stick. If the USB stick has been removed during a run, data can be retrieved manually from the instrument.

3.4 PyroMark Q24 MDx Instrument

1. Instrument lid
2. Screen
3. Menu buttons
4. USB port



5. Power switch
6. LED is lit when the cooling device is receiving power
7. USB port (inactive)
8. Light button for the coolant level window
9. Window showing the coolant level
10. Instrument power connector 24V ⎓
11. Cooler power connector 12V ⎓

PyroMark Q24 Instrument exterior.

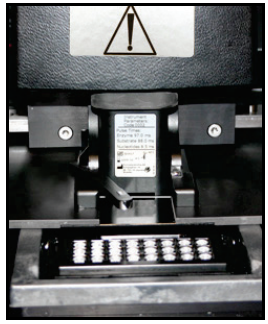
3.4.1 Process chamber

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 the PyroMark Q24 MDx Instrument 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 rapid mixing of samples and reagents in the plate, the heating block inside the process chamber is constantly vibrated during the run.

3.4.2 Dispensing unit



Dispensing unit.

A reagent cartridge (the PyroMark Q24 Cartridge) filled with the required volumes of PyroMark Gold Q24 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 PyroMark Q24 Plate and reagents are dispensed in a zigzag fashion by a pneumatic system.

3.5 Sample preparation with the PyroMark Q24 MDx Vacuum Workstation

The DNA to be analyzed is amplified by PCR using 2 primers, one of which is biotinylated. The biotinylated PCR product is then immobilized on streptavidin-coated Sepharose® beads.

Samples to be analyzed using the PyroMark Q24 MDx Instrument should be prepared according to the instructions in Section 5.3, using the PyroMark Q24 MDx Vacuum Workstation.

3.6 Analysis software

The PyroMark Q24 MDx is shipped with PyroMark Q24 MDx Software.

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

- Microsoft® Windows® 7 (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

To view reports generated in PDF format, a PDF reader must be installed on the computer. Adobe® Reader® can be downloaded at www.adobe.com.

4 Installation Procedures

4.1 System delivery and installation

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

The following items are delivered:

- PyroMark Q24 MDx Instrument (including two USB sticks)
- PyroMark Q24 MDx Vacuum Workstation (purchased separately)
- *PyroMark Q24 MDx User Manual*
- *PyroMark Q24 MDx Software User Guide*
- PyroMarkQ24 Plate Holder

Reagents and other accessories can be ordered separately, visit www.qiagen.com/products/PyromarkQ24MDx.aspx.

4.2 Requirements

Site

The PyroMark Q24 MDx Instrument and PyroMark Q24 MDx 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 MDx Instrument and PyroMark Q24 MDx 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 MDx Instrument with the lid open. Allow at least 10 cm (4 in.) of free space behind the instrument for cabling.

Remove the foam dispensing module transport lock. Keep the transport lock for future transportation of the instrument.

The PyroMark Q24 MDx Instrument must be placed within approximately 1.5 m (59 in.) of two properly grounded (earthed) AC power outlets. The power lines to the PyroMark Q24 MDx Instrument should be voltage regulated and surge protected.

Note: We recommended plugging the instrument directly into its own power outlets and not to share the power outlets with other lab equipment. Do not place PyroMark Q24 MDx on a vibrating surface or near vibrating objects.

CAUTION	**Risk of overheating** To ensure proper ventilation, maintain a minimum clearance of 10 cm at the sides and rear of the PyroMark Q24 MDx Instrument. Slits and openings that ensure the ventilation of the PyroMark Q24 MDx Instrument must not be covered.

Power requirements

The PyroMark Q24 MDx Instrument operates at:

- Input 100–240 V AC, 50–60 Hz, 160 VA
- Instrument rating 24 V DC, 40 W
- Cooler rating 12 V DC, 60 W

The PyroMark Q24 MDx Vacuum Workstation operates at:

- 100 V AC, 50/60 Hz, 25 VA
- 115 V AC, 60 Hz, 25 VA
- 230 V AC, 50 Hz, 25 VA

Make sure that the voltage rating of the PyroMark Q24 MDx 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 MDx Instrument must be correctly grounded (earthed). The PyroMark Q24 MDx Instrument is equipped with two 3-conductor AC power cords. To preserve this protection feature, do not operate the PyroMark Q24 MDx Instrument from AC power outlets that have no ground (earth) connection.

4.3 Installation of the analysis software

4.3.1 Installing or upgrading PyroMark Q24 MDx Software

Note: If the computer is connected to a network, network policy settings may prevent you from completing this procedure. For more information, contact your system administrator.

1. Ensure that the computer meets the minimum requirements; see Section 3.6.
2. Close any programs running on the computer.
3. Insert the PyroMark Q24 MDx Software CD into the CD drive.
4. In the CD menu, click "Install PyroMark Q24 MDx Software".

   If the CD menu does not appear automatically:
   - Select "(My) Computer" in the Windows "Start" menu.
   - Right-click the CD drive with the software CD and select "Open".
   - Double-click the file **autorun.exe**.
5. Follow the instructions that appear in the "Setup Wizard".

   Note: If .NET Framework 3.5 has to be installed (installation is prompted by the "Setup Wizard"), the installation has to be restarted when the .NET Framework installation has been completed, i.e., open the CD menu

(see step 4) and click "Install PyroMark Q24 MDx Software".

6. When the software has been successfully installed, click "Exit Setup" in the CD menu.
7. Please use Windows Update (www.update.microsoft.com) to check for any critical updates to the .NET Framework 3.5.

To view reports generated by PyroMark Q24 MDx Software in PDF format, a PDF reader must be installed on the computer. Adobe Reader can be downloaded at www.adobe.com.

4.3.2 Uninstalling PyroMark Q24 MDx Software

1. Select "Control Panel" in the Windows "Start" menu.
2. In the "Control Panel", click "Uninstall a program" under the Programs category.
3. In the list of programs, select "PyroMark Q24".
4. Click "Uninstall".
5. Repeat steps 3 and 4 for PyroMark Launcher.

5 Operating Procedures

This section describes how to operate PyroMark Q24 MDx.

Before proceeding, we recommend that you familiarize yourself with the features of PyroMark Q24 MDx Instrument by referring to Section 3.4.

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 processing, select "Administration" in the main menu.
2. Select "Set Date and Time" using the ▲ and ▼ screen buttons and press "OK".
3. Select the parameter you want to edit using the ◀ 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 one of the USB sticks supplied into the USB port at the front of the instrument.
2. Using the ▲ and ▼ screen buttons, select "Administration" in the main menu and press "OK".
3. Select "Copy Unsaved Runs" and press "OK".
4. Using the ▲ and ▼ screen buttons, select the run file for retrieval 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 memory.

Note: When space becomes insufficient, the run files are deleted in chronological order. Files that have never been saved to a USB stick (see Section 5.1.2) will not be deleted.

Copy recently saved runs as follows:

1. When the instrument is not processing, insert one of the USB sticks supplied into the USB port at the front of the instrument.
2. Using the ▲ and ▼ screen buttons, select "Administration" in the main menu and press "OK".
3. Select "Copy Recently Saved Runs" and press "OK".
4. Using the ▲ and ▼ screen buttons, select the run file for retrieval 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 processing, insert one of the USB sticks supplied into the USB port at the front of the instrument.
2. Using the ▲ and ▼ screen buttons, select "Administration" in the main menu 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 switched off during a run), extract run files as follows:

1. When the instrument is not processing, insert one of the USB sticks supplied into the USB port at the front of the instrument.
2. Using the ▲ and ▼ screen buttons, select "Administration" in the main menu 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 Viewing acknowledgements, version, and contact information

View acknowledgements, 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".

5.1.7 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. 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. Using the ▲ and ▼ screen buttons, select "Administration" in the main menu and click "OK".
4. Select "Upgrade Software" and click "OK".
5. Follow the instructions on the screen.

5.1.8 Run an external application

The "Run External Application" menu option is used for service applications. Only run a service application when instructed to do so 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 be found in the *PyroMark Q24 MDx Software User Guide*.

5.2.1 Starting PyroMark Q24 MDx Software

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

The *PyroMark Q24 MDx Software User Guide* can be accessed at any time by pressing the "F1" key when in the software.

5.2.2 Setting up an assay

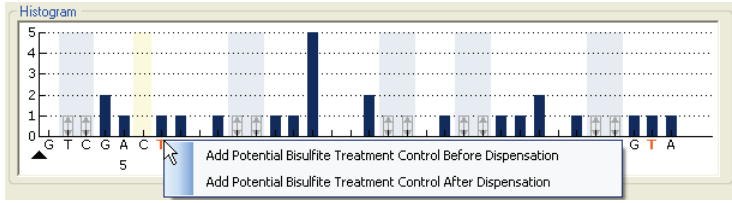
1. In the shortcut browser, right-click the folder you want to place the assay file in and select "New Assay" followed by the desired assay type (AQ, CpG, or SQA) from the context menu.
   Note: To add a shortcut to a folder in the shortcut browser, click "Add Folder Shortcut".
2. Enter the file name and press "Enter".
3. If creating an AQ or CpG assay, type or paste the "Sequence to Analyze" and then click the "Generate Dispensation Order" button. If creating an SQA assay, enter the "Dispensation Order".
4. Click in the toolbar.
   Note: Before running your samples, validate your assay using a reference DNA sample; see Appendix B.
   Note: When using QIAGEN kits, use the settings stated in the kit handbooks.

Optional

If desired, enter a note about the assay and set up the variable positions in the "Variable Positions" tab (AQ and CpG assays only).

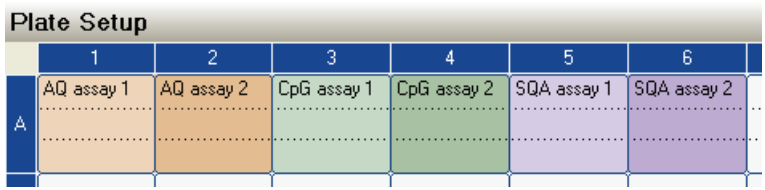
If creating a CpG assay, it is recommended that bisulfite treatment controls are added. In the sequence before bisulfite treatment, check if the suggested bisulfite controls are Cs converted to **T**s (read as Gs and **A**s in a reverse assay) and are suitable as controls, or not.

To add a control, left-click a bold, orange **T** or **A** in the histogram, preferably at the beginning of the sequence.

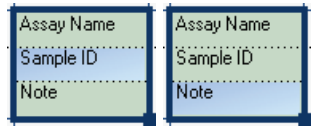


5.2.3 Setting up a run

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.
   Note: 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 Section 5.2.4 for detailed instructions.
4. Add an assay to each well used, e.g., drag an assay from the shortcut browser to a well or a selection of wells.
   A well is colored according to the assay loaded into the well.



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.



6. Click in the toolbar.
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.

   The run file can now be processed by inserting the USB stick into the USB port at the front of the PyroMark Q24 MDx Instrument (see Section 5.5).

Optional

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

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 *PyroMark Q24 MDx Software User Guide* (press the "F1" key when in PyroMark Q24 MDx 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. Only the run setup, not the run and analysis data, will be copied.

5.2.4 Managing instrument methods

The instrument method should be selected according to the reagents and reagent cartridge that will be used for the run. The method number printed on the PyroMark Q24 Cartridge corresponds to specific method settings provided at www.qiagen.com/Products/PyroMarkQ24MDx.aspx.

Note: It is recommended that only methods supplied by QIAGEN are used.

To import a new method:

1. From the Web site above, download the method file corresponding to the method number printed on the cartridge label. Save it to the computer running the PyroMark Q24 MDx Software.
2. In the "Instrument Methods" dialog box, click "Import". The "Find Instrument Method" dialog box opens.
3. Locate and select the downloaded method and click "Open".

To create a new method:

1. In the "Instrument method" dialog box, select an existing method and click "Save As".
2. Enter a name for the new method and press "Enter".
3. Change the method settings in the dialog box to match those posted on www.qiagen.com/Products/PyroMarkQ24MDx.aspx.
4. Click "Save".

Method parameters

In the "Instrument Methods" dialog box, the following parameters are available.

Reagent pressure	Pressure (millibar) for dispensation of the enzyme mix and substrate mix.
Enzyme pulse time	Dispensation time (milliseconds) for the enzyme mix.
Substrate pulse time	Dispensation time (milliseconds) for the substrate mix.
Nucleotide pressure	Pressure (millibar) from the dispensation of nucleotides.
Nucleotide pulse time	Dispensation time (milliseconds) for nucleotides.
Note	Note about the instrument method (optional).

5.3 Sample preparation

Samples to be analyzed using the PyroMark Q24 MDx Instrument 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 MDx Vacuum Workstation
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80°C
- PyroMark Q24 Plate
- 24-well PCR plate or strips

- PyroMark Q24 Cartridge
- Strip caps
- Streptavidin Sepharose High Performance (34 µm, 5 ml, GE Healthcare; see www.gelifesciences.com)
- QIAGEN IVD-labeled assays for Pyrosequencing
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- Ethanol (70%)
- PyroMark Binding Buffer
- PyroMark Denaturation Solution
- PyroMark Wash Buffer concentrate
- PyroMark Annealing Buffer

5.3.1 PyroMark Q24 MDx Vacuum Workstation function test

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

1. Add 100 µl high-purity water to each well of a 24-well PCR plate.
2. Fill a trough with 70 ml high-purity 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 not, 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 to be analyzed by PCR using one of the primers biotinylated. To receive valid analysis data, see Appendix B.

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 µl per sample) and Binding Buffer (40 µl per sample) in a tube. Add high-purity water to a total volume of 80 µl per well — including the PCR product to be added in step 4.
   The amount of water depends on the amount of PCR product used. For example: If using 15 µl of PCR product, 2 µl of beads, and 40 µl of Binding Buffer, 23 µl of high-purity water must be added.
3. Add the solution prepared in step 2 to a 24-well PCR plate or strips.
4. Add 5–20 µl of a well-optimized, biotinylated PCR product to each well of the PCR plate (or strips) according to the plate setup (see Section 5.2.3).
   Note: When using the PyroMark PCR Kit, 5–10 µl of the PCR product gives satisfactory Pyrosequencing results in most cases. This volume should be adjusted to achieve single peak heights of at least 40 RLU in the Pyrogram.
   Note: The total volume per well should be 80 µl.
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 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 into the PyroMark Q24 Plate

WARNING	**Hazardous chemicals**
	The Denaturation Solution used with the vacuum workstation contains sodium hydroxide, which is irritating to eyes and skin. Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the 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.

* 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).

Things to do before starting

Prewarm one of the supplied PyroMark Q24 Plate Holders to be used in Section 5.3.5 by placing it (without a plate) on a heating block at 80°C.

Procedure

1. Ensure that the PyroMark Q24 MDx 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 filter probes should be replaced after preparation of approximately 100 plates.

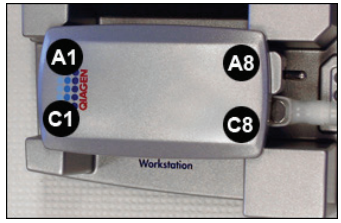
Note: Empty the waste container if necessary (see Section 5.6.2).

2. Fill five separate troughs supplied with the PyroMark Q24 MDx Vacuum Workstation as follows:
   - Approximately 50 ml ethanol (70%) (1)
   - Approximately 40 ml Denaturation Solution (2)
   - Approximately 50 ml 1x Wash Buffer (3)
   - Approximately 50 ml high-purity water (4)
   - Approximately 70 ml high-purity water (5)

   A suggested setup is shown in the following image. 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 high-purity water. Flush the probes with 70 ml high-purity 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. Close the vacuum switch on the tool (Off) and place the tool in the Parking position.
7. Refill trough 5 with 70 ml high-purity water.
8. Dilute the sequencing primer to 0.3 µM in Annealing Buffer. Add 25 µl of the solution 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. Immediately after immobilization (see Section 5.3.3), place the PCR plate (or the strips) and PyroMark Q24 Plate on the worktable. Ensure that the plate is in the same orientation as when the samples were loaded; see image below for guidance.



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 template. Hold the filter 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. Flush the filter probes for 5 s.
14. Transfer the tool to the trough containing Denaturation Solution. Flush the filter probes for 5 s.
15. Transfer the tool to the trough containing Wash Buffer. 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 following image).

17. While holding the tool over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).
18. Release the beads in the plate containing sequencing primer, by shaking the tool gently from side to side.
19. With the vacuum switch closed (Off), transfer the tool to the trough containing high-purity water and agitate the tool for 10 s.
20. Wash the filter probes by lowering the probes into the second trough of high-purity water and applying vacuum. Flush the filter probes with 70 ml high-purity 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 MDx Vacuum Workstation should be checked for dust and spillage, (see Section 5.6.2).

5.3.5 Annealing of sequencing primer to samples

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

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

5.4 Preparation of PyroMark Gold Q24 Reagents

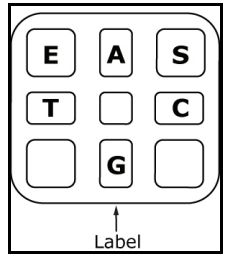
WARNING	**Sharp needles** Do not touch the sharp needles at the bottom of the reagent cartridge. Handle the needles with care. Small particles and fibers may obstruct the needles.

1. Open the PyroMark Gold Q24 Reagents box and remove the vials containing freeze-dried enzyme and substrate mixtures, and the tubes containing nucleotides.
2. Reconstitute the volumes of reagents required and fill PyroMark Q24 Cartridge according to the handbook 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. It is recommended that the reagent cartridge is used a maximum of 30 times. If the reagent cartridge has not been used in 4 weeks or longer (e.g., it has been stored), clean the cartridge and check that it can be used for

analysis by performing the function test (steps 4–6 in Section 5.5.5).



PyroMark Q24 Cartridge compartments from above.

5.5 Processing a run on the PyroMark Q24 MDx Instrument

The lid of the PyroMark Q24 MDx Instrument 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** To avoid contact with moving parts during operation of the PyroMark Q24 MDx Instrument, 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 MDx Instrument, contact QIAGEN Technical Services immediately.

WARNING 	**Sharp needles** Do not touch the sharp needles at the bottom of the reagent cartridge. Handle the needles with care. Small particles and fibers may obstruct the needles.

5.5.1 Instrument software

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

Runs are started and monitored through the instrument software. 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 run 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 instrument needs to be disconnected quickly from mains power.
2. Switch on the instrument. The power switch is located at the rear of the instrument (see image in Section 3.4).

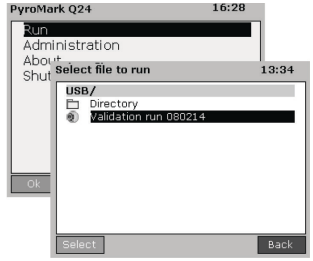
5.5.3 Starting the run

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 (see image below).
3. Ensure the cartridge is properly inserted, with the line visible in front of the cartridge, and close the gate.
4. Open the plate-holding frame and place the plate on the heating block inside the instrument.
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.
2. Using the ▲ and ▼ screen buttons, select "Run" in the main menu 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 of the Pyrogram area.

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 ensure that the dispensation capillaries are flushed and filled with solution.
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 7.3.

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. If the reagent cartridge is to be reused, clean it according to the instructions below.
10. If this was the last run for the day, follow the instructions in Section 5.6.

Note: Be sure to observe all national, 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.

WARNING	**Sharp needles**
	Do not touch the sharp needles at the bottom of the reagent cartridge. Handle the needles with care. Small particles and fibers may obstruct the needles.

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

The following items are required:

- Powder-free gloves
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- Beaker (not always required)
- Lint-free tissues.

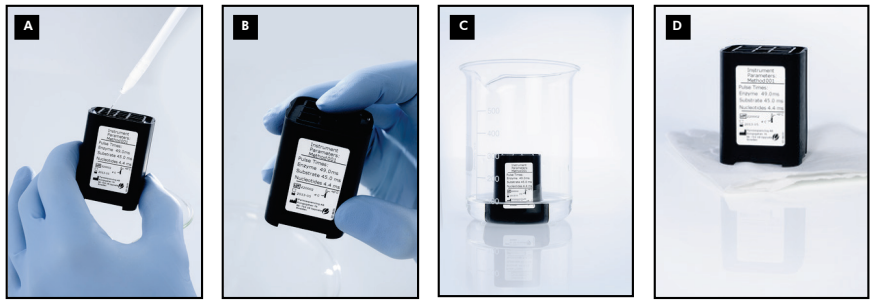
To clean and check that the reagent cartridge can be used for analysis:

1. Discard any solutions remaining in cartridge.
2. Rinse the cartridge compartments 4 times with high-purity water.
3. Spray the outside of the needles using high-purity water.
4. Check that the cartridge needles are not blocked or damaged. Completely fill the compartments with high-purity water. Hold the cartridge over a sink or beaker while pressing firmly on top of each compartment with a finger (wear powder-free gloves). A jet of water should come straight out of the tip of each needle.

5. If a needle is blocked, follow step 5a. If the jet of water comes out of a needle at an angle (not parallel to the direction of the needle), follow step 5b. If all nee3dles are working properly, proceed to step 6.

5a. If a needle is blocked (for example, if the reagent cartridge was left overnight without cleaning), fill the compartments with high-purity water, and immerse the cartridge in a beaker with enough high-purity water to cover the needles. Leave the cartridge in the beaker for 1 h, rinse it, and repeat step 4. Proceed to step 6.

5b. If the jet of water comes out at an angle, refill the compartment with water and repeat step 4. If the water still comes out at an angle, discard the cartridge.



Cleaning procedure for PyroMark Q24 Cartridge.

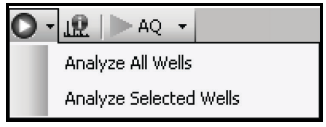
- **A** Filling cartridge compartments with high-purity water
- **B** Checking for blocked or damaged needles
- **C** Cleaning blocked needles
- **D** Drying the cartridge on a lint-free tissue

6. When all needles have been rinsed and tested, discard the water and leave the reagent cartridge to dry on a lint-free tissue.
7. When the reagent cartridge is dry, store the reagent cartridge in a PyroMark Q96 HS Tip Holder Box to protect it from dust and (sun)light.

5.5.6 Analyzing the run

Detailed instructions for analyzing the run are available in the *PyroMark Q24 MDx Software User Guide* (press the "F1" key when in PyroMark Q24 MDx Software).

1. Move the processed run file from the USB stick to a computer running PyroMark Q24 MDx Software.
2. Open the run file by double-clicking the run file ( ) in the shortcut browser. If several assay types are included, select analysis mode in the dialog box that opens.
   To add a shortcut to a file or folder in the shortcut browser, click "Add File Shortcut" or "Add Folder Shortcut".
3. In the "Overview" tab, either analyze all wells or a selection of wells with a valid analysis setup for the current analysis mode.



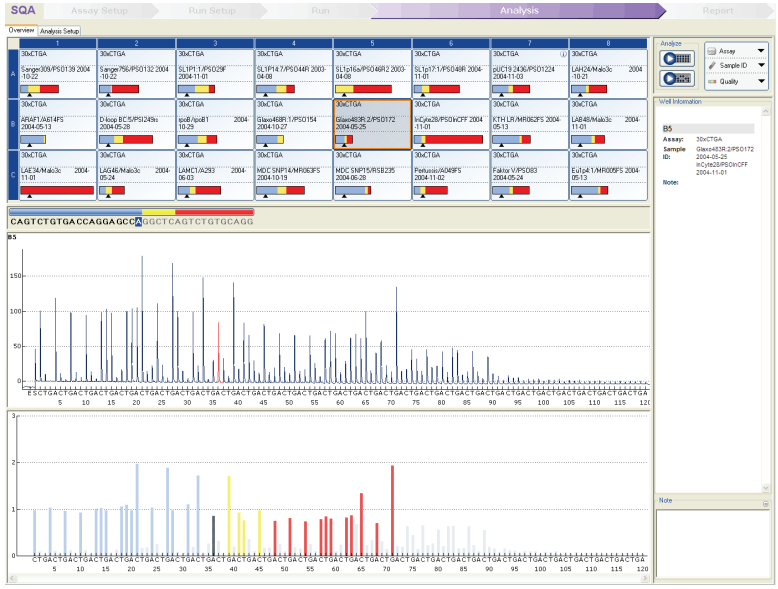
Analysis modes

PyroMark Q24 MDx Software has three analysis modes: AQ, CpG, and SQA. To toggle between the modes, select "AQ", "CpG", or "SQA" in the toolbar. Genotyping of SNPs and InDels can be accessed from the "Reports" menu in the AQ mode.

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 in the "Overview" tab, the corresponding Pyrogram is displayed in the Pyrogram area and the well information (including analysis warnings) is listed in the "Well Information" area.



Quality assessments

The plate overview in the "Overview" tab gives a quick overview of the quality assessments.

: Shows the quality assessment of all variable positions in the well or of all the bases in the base-called sequence.

: Shows the quality assessment at the end of the quality control window (SQA assays only).

Quality colors

Blue: Passed

Yellow: Check

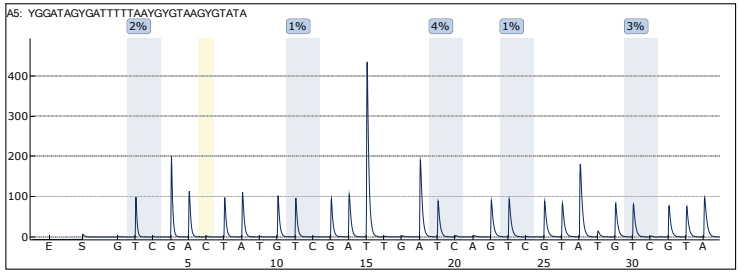
Red: Failed

White: Not analyzed*

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

AQ analysis results

The allele frequencies are displayed in Pyrogram, for example A: 96% G: 4% and --: 56% AT: 44% (InDel). The quality assessment is displayed by the background color of the result.



Example Pyrogram for a CpG assay. Variable positions in AQ and CpG assays are highlighted with a blue-gray background color, and bisulfite treatment controls in CpG assays with a light yellow background color.

CpG analysis results

The methylation percentages are displayed in Pyrogram, for example 96%. The quality assessment is displayed by the background color of the result.

A methylation bar in the plate overview shows the methylation level for each CpG site in the well.

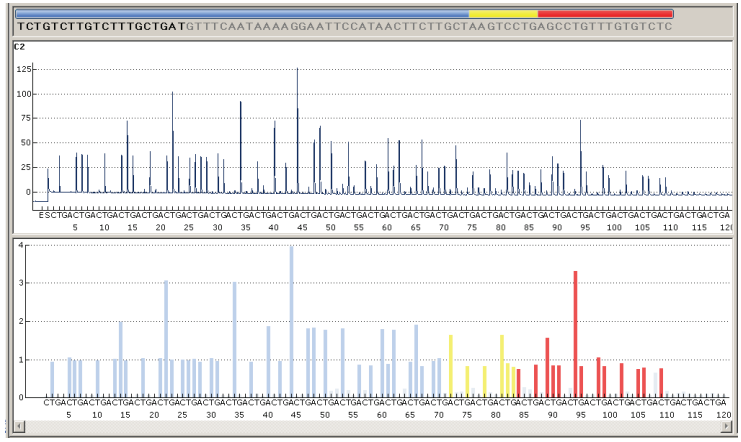
Light green: Below the expected range

Green: Within the expected range

Dark green: Above the expected range

SQA analysis results

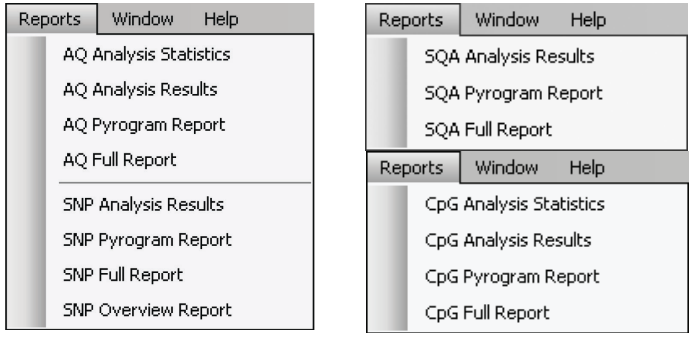
The base-called sequence is displayed in the "Overview" tab. The bases in the base-called sequence and the peaks in the compensated Pyrogram are colored according to their quality assessments.



Example of a base-called sequence and Pyrogram for a SQA assay.

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 "View, Print, and Save Analysis Reports" section of the *PyroMark Q24 MDx Software User Guide* (press the "F1" key when in PyroMark Q24 MDx Software).



To view reports generated in PDF format, a PDF reader must be installed on the computer. Adobe Reader can be downloaded at www.adobe.com.

5.6 Finishing work and shutting down

5.6.1 Shutting down the instrument

1. When the instrument is not processing, using the ▲ and ▼ screen buttons, select "Shutdown" from the main menu 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**
	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 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.

* 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 national, state, and local environmental regulations for the disposal of laboratory waste.

The following item is required:

- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)

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 high-purity water, or replace them, if necessary.
4. Empty the waste container.
   Note: The cap can be removed without disconnecting the tubing.

5. If the vacuum workstation must be cleaned (e.g., from dust or spillage), follow the instructions in Section 6.3.

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 (see image below) and check that the coolant level is visible in the window. If it is not, contact QIAGEN Technical Services.

5.7 Backup of PyroMark Q24 files

The data generated by PyroMark Q24 MDx 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.

6 Maintenance

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

- Regular performance checks
- Cleaning of the instrument

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

Before undertaking maintenance procedures, it is recommended that you familiarize yourself with the safety information by referring to Section 1.

Important: Disconnect the instrument from mains power before cleaning.

Servicing

QIAGEN offers comprehensive Service Support Agreements, including Warranty Extensions, Full Cover Support Agreements, and system/application training, including on-site installation and annual preventative maintenance. Service Support Agreements maximize productivity and ensure high performance from your system. 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 MDx

Check that PyroMark Q24 MDx is functioning according to specifications by measuring imprecision, bias, and linearity for an AQ or CpG assay using PyroMark Q24 Validation Oligo.

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

6.2 Maintenance of the PyroMark Q24 MDx Instrument

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%)
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- Clean, non-abrasive, lint-free cloths

Procedure

1. When the instrument is not processing, using the ▲ and ▼ screen buttons, select "Shutdown" in the main menu 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.
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 inside the instrument, 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, using the ▲ and ▼ screen buttons, select "Shutdown" in the main menu 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.
6. Clean each well hole/light guide carefully using cotton swabs lightly moistened with 70% ethanol (see image, next page).
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, next page).
8. Close the plate-holding frame and the instrument lid and reconnect the instrument to the mains power.

CAUTION	**Light guide maintenance** Use lint free tissues to clean the space between the heating block and the light guide block inside the instrument. Do not use paper tissues.

6.3 Maintenance of the PyroMark Q24 MDx Vacuum Workstation

WARNING	**Hazardous chemicals** [W6]
	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 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.

* 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 MDx 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
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- 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 trough the filter probes, all probes should be replaced after preparation of approximately 100 plates.

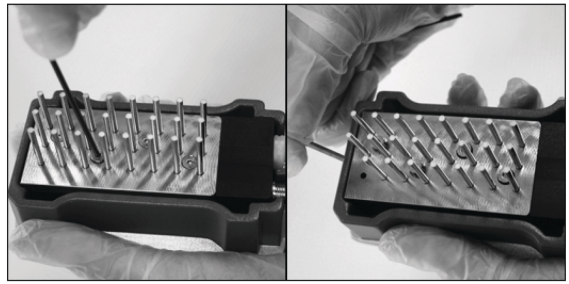
Note: Use gloves (powder-free) to avoid contaminating the filter probes.

The following items are required:

- Powder-free gloves
- 2 mm Allen key (supplied with the system)
- High-purity water (Milli-Q 18.2 MΩ x cm or equivalent)
- New filter probes (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. 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 (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 properly 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 national, state, and local environmental regulations for the disposal of laboratory waste.

The following items are required:

- New tubing (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 national, 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, cat. no. 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.

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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 Field Service Specialist in solving the problem.

When calling QIAGEN Technical Services 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 (in the "Run Information" report) 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 or right-click the file in the shortcut browser and select "Run Information" from the context 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 speed 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 file:

- Select "Export Environment Data" from the "Tools" menu
- Select the destination folder for the data file 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
a)	PCR failed due to low DNA quality	Check the PCR samples using an agarose gel to confirm there is one strong specific band. If not, rerun PCR with high-quality DNA. The PyroMark PCR Kit is recommended for highly specific amplification of bisulfite-converted DNA and genomic DNA from various sources.
b)	Poorly optimized PCR	Check the PCR samples using an agarose gel to confirm there is 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.
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.

		Comments and suggestions
f)	Too much PCR product depletes substrate, leading to missing peaks at the end of the sequence	Use less PCR product.
g)	One or several of the compartments in the reagent cartridge were not correctly filled	Be sure to add sufficient reagents (open the run setup and select "Pre Run Information" from the "Tools" menu). Follow the instructions provided in the handbooks supplied with the products.
h)	One of the needles in the reagent cartridge is blocked or damaged (noted as a missing presequencing signal and/or missing peaks in Pyrogram)	In case of bent needles, discard the reagent cartridge. Be sure to observe all national, state, and local environmental regulations for disposal of laboratory waste.
i)	Reagents incorrectly dissolved or stored	Be sure to follow the instructions in the handbook supplied with PyroMark Gold Q24 Reagents. Include an empty well (Annealing Buffer only) in the run setup to check whether background peaks are coming from nucleotides.
j)	Low signal due to dirty light guides	Clean the heating block and light guides; see Section 6.2.2.
k)	Incorrect sequence to analyze	Correct the sequence to analyze and, if necessary, rerun the samples.
l)	Background peaks in Pyrogram	Follow the recommendations in Appendix B the first time an assay is run. Redesign the assay.

	Comments and suggestions
m) 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).
n) Crosstalk (light from one well appears in the neighboring well)	Avoid placing assays with high signals close to assays with low signals.
o) Dispensation error	Replace the reagent cartridge. If the problem remains, contact QIAGEN Technical Services.
p) 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.
q) dUTP used in the PCR reaction	Replace dUTP with dTTP since the A nucleotide used in Pyrosequencing reactions binds less stringently to dUTP.
r) Plus shift	Change the dispensation order.
s) 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 Guide section of the *PyroMark Q24 MDx Software User Guide*.

7.3 Instrument-related errors

Error messages	Comments and suggestions
a) Too many unsaved runs in the instrument. Please go to folder "Unsaved Runs" and save them to USB memory	Transfer unsaved runs to a USB stick; see Section 5.1.2.
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: ■ 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 MDx Software.
d) Could not copy "file" to USB memory	Try another USB stick. We recommend using the USB sticks supplied by QIAGEN.
e) Failed to connect to the hardware/the connection to the hardware is lost, please restart the instrument	Restart the instrument. If the problem remains, contact QIAGEN Technical Services.
f) No valid upgrade folders/files found on USB memory	Ensure your upgrade installation files are located in a folder called "Upgrade" at the root of the USB stick.

Note: For all other instrument error messages, please contact QIAGEN Technical Services.

		Comments and suggestions
Other problems		
a)	The instrument is making unexpected noise when starting	Check that the reagent cartridge is inserted correctly.
b)	No contact with the USB stick	The used USB stick is damaged or not compatible with the system. It is recommended that USB sticks supplied by QIAGEN are used.
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	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. Ensure that the tubing is connected correctly and that there is no leakage. The waste filter may be wet and require replacing; see Section 6.3.5.
b)	Vacuum lost during sample preparation	Ensure that the tubing is connected correctly and there is no leakage. The waste filter may be wet and require replacing; see Section 6.3.5.
c)	Filter probes not working properly	Ensure filter probes are working properly; see Section 6.3.2.
d)	Liquid left in some wells in the immobilization plate	Replace the corresponding filter probes; see Section 6.3.2.

		Comments and suggestions
e)	White remains (Sepharose beads) in the immobilization plate	If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.

7.5 Verification of correct installation and operation

PyroMark Control Oligo is sold together with the PyroMark Q24 MDx and required to verify proper installation and operation of the system. The PyroMark 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 PyroMark Control Oligo, see the *PyroMark Q24 Validation Oligo Handbook*, supplied with the product.

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8 Glossary

Term	Description
AQ	Analysis mode used for quantification of different alleles.
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 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.
CpG	Analysis mode used for analysis of CpG methylation.
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.

Term	Description
Drop off	A continual decrease in peak height normally seen in the Pyrogram.
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.
InDels	Insertion and/or deletions.
Instrument methods	A method that describes physical settings for the instrument, such as mixer speed, block temperature, and pulse time settings.

Term	Description
IUPAC	International Union of Pure and Applied Chemistry. An organization providing recommendations on organic and biochemical nomenclature, symbols, terminology, etc.
	IUPAC codes
	A = Adenine C = Cytosine G = Guanine T = Thymine R = Purine (A or G) Y = Pyrimidine (C or T) M = C or A K = T or G W = T or A S = C or G B = C, T, or G (not A) D = A, T, or G (not C) H = A, T, or C (not G) V = A, C, or G (not T) N = Any base (A, C, G, or T)
Out of phase	When one of the alleles is sequenced ahead of the other.
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" in the Pyrogram), 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. Incorporated nucleotides are shown as peaks in the Pyrogram.

Term	Description
Quality control window	Gives an overview of the quality at the end of a defined number of bases in the base-called sequence. A setting in the SQA analysis mode.
Reference peak	Nonvariable peaks (i.e., peaks that are not a part of a variable position) are referred to as "reference peaks". Reference peaks are used in the analysis both as references when calculating the single peak height level, 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 should be used for preparation of biotinylated PCR products.
SQA	Analysis mode used for base-calling of unknown sequences.
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 DNA polymerase.
Shift	Plus 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. Minus shift: A small proportion of the template sequences that fails to incorporate a nucleotide will be sequenced subsequent to the rest of template.

Term	Description
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.
Variable position	A region in the sequence that varies at one or more variable bases. In PyroMark Q24 MDx Software, the variable positions are highlighted with a blue-gray background color in the histogram and Pyrogram.

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Appendix A

Technical data

QIAGEN reserves the right to change specifications at any time.

Environmental conditions

Operating conditions: PyroMark Q24 MDx Instrument

Input power	100–240 VAC, 50–60 Hz
Input power consumption	Maximum 160 VA
Instrument rating	24 V DC, 40 W
Cooler rating	12 V DC, 60 W
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)

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 MDx Vacuum Workstation

Power	100 V AC, 50/60 Hz or 115 V AC, 60 Hz or 230 V AC, 50 Hz Power consumption: Maximum 25 VA
Overvoltage category	II
Air temperature	15–32°C (59–90°F)
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.) (includes filled troughs and a full waste container)
Capacity	Up to 24 samples per plate
Chemical resistance	pH 4 to pH 9, common detergents, 0.5 M sodium hydroxide, 70% ethanol

PyroMark Q24 MDx Software

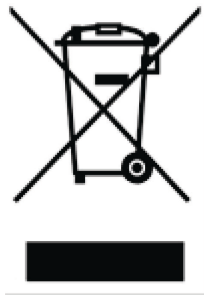
Operating system	Microsoft Windows 7, English version
Processor	Intel Pentium IV, 3 GHz (or higher)
RAM	1 GB
Free hard disk space	100 MB
Graphics card	Supporting the resolution of the monitor
Monitor	1280 x 1024 pixels
Pointer device	Mouse or similar
Interfaces	USB port and CD-ROM

Waste Electrical and Electronic Equipment (WEEE)

This section provides information about disposal of waste electrical and electronic equipment by users.

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 laws and regulations.

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.



Recycling can be provided by QIAGEN upon request at additional cost. In the European Union, in accordance with the specific WEEE recycling requirements and where a replacement product is being supplied by QIAGEN, free recycling of its WEEE-marked electronic equipment is provided.

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.

FCC declaration

The ''United States Federal Communications Commission'' (USFCC) (in 47 CRF 15. 105) declared that the users of this product must be informed of the following facts and circumstances.

''This device complies with part 15 of the FCC:

Operation is subject to the following two conditions: (1) This device may not cause harmful interference, and (2) this device must accept any interference received, including interference that may cause undesired operation.''

"This Class B digital apparatus complies with Canadian ICES-0003.''

The following statement applies to the products covered in this manual, unless otherwise specified herein. The statement for other products will appear in the accompanying documentation.

Note: This equipment has been tested and found to comply with the limits for a Class B digital device, pursuant to Part 15 of the FCC Rules and meets all requirements of the Canadian Interference-Causing Equipment Standard ICES-003 for digital apparatus. These limits are designed to provide reasonable protection against harmful interference in a residential installation. This equipment generates, uses, and can radiate radio frequency energy and, if not installed and used in accordance with the instructions, may cause harmful interference to radio communications. However, there is no guarantee that the interference will not occur in a particular installation. If this equipment does cause harmful interference to radio or television reception, which can be determined by turning the equipment off and on, the user is encouraged to try to correct the interference by one or more of the following measures:

- Reorient or relocate the receiving antenna.

- Increase the separation between the equipment and receiver.
- Connect the equipment into an outlet on a circuit different from that to which the receiver is connected.
- Consult the dealer or an experienced radio/T.V. technician for help.

QIAGEN GmbH Germany is not responsible for any radio television interference caused by unauthorized modifications of this equipment or the substitution or attachment of connection cables and equipment other than those specified by QIAGEN GmbH, Germany. The correction of interference caused by such unauthorized modification, substitution or attachment will be the responsibility of the user.

EC declaration of conformity

Name and address of the legal manufacturer

QIAGEN GmbH
QIAGEN Strasse 1
40724 Hilden
Germany

An up-to-date Declaration of Conformity can be requested from QIAGEN Technical Services.

Appendix B

Assay design and validation

Assay design

Pyrosequencing assays can be designed using the latest version of PyroMark 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. Ensure you use the correct assay type in PyroMark ADSW. We recommend the use of QIAGEN IVD-labeled assays for Pyrosequencing, which include all necessary optimized primers.

PCR

For PCR amplification we recommend using PyroMark PCR Kit (QIAGEN) which is specifically optimized for Pyrosequencing analysis and enables highly specific and unbiased amplification of template DNA for various Pyrosequencing applications, such as mutation detection, SNP analysis, methylation analysis, and base-calling. The convenient master mix format enables specific amplification of various starting materials such as genomic DNA from a variety of species, as well as bisulfite converted DNA, using only one protocol.

PCR primers

One of the primers must be biotin-labeled to enable immobilization to streptavidin-coated beads (Streptavidin Sepharose High Performance; GE Healthcare) during the preparation of a single-stranded DNA template. The orientation of the assay can either be forward or reverse. If designing primers with PyroMark ADSW, the primer that needs to be biotinylated is indicated.

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 the streptavidin-coated Sepharose beads.

Amplicon length

The optimal amplicon length for Pyrosequencing assays is between 80 and 200 bp, although products up to 500 bp may work well. Amplicons for CpG assays should ideally be shorter than 200 bp.

Sequencing primer

Design sequencing primers using PyroMark ADSW. When designing an InDel assay, it is highly recommended that the sequencing primer is located a few bases before the variable position. We recommend the use of QIAGEN IVD-labeled assays for Pyrosequencing, which include all necessary optimized primers.

PCR setup

PCR reactions of 25 µl are set up using the PyroMark PCR Kit. Ensure that you follow the instructions provided in the *PyroMark PCR Handbook*.

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.

Starting template

The yield and quality of PCR product is affected by both the quality and quantity of the nucleic acid starting template. This is particularly true for amplification of long regions from DNA that has been fragmented by bisulfite-treatment or extracted from paraffin-embedded material.

Quality of starting template

Since PCR consists of multiple rounds of enzymatic reactions, it is more sensitive to impurities such as proteins, phenol/chloroform, salts, ethanol, EDTA, and other chemical solvents than single-step enzyme-catalyzed processes. QIAGEN offers a complete range of nucleic acid preparation systems, ensuring the highest-quality templates for PCR. These include the QIAprep® system for rapid plasmid purification, the QIAamp® and DNeasy® systems for rapid purification of genomic DNA and viral nucleic acids, and the RNeasy® system for RNA preparation from a variety of sources. For more information about QIAprep, QIAamp, DNeasy, and RNeasy products, contact one of our Technical Service Departments (see back cover) or visit www.qiagen.com.

Quality of starting template when performing CpG assays

Critical parameters for a successful PCR using bisulfite-treated DNA templates include complete bisulfite conversion and DNA fragments that are long enough for PCR. EpiTect® Bisulfite Kit provides a fast and reliable procedure for efficient bisulfite conversion and a unique DNA Protect Buffer prevents DNA fragmentation during the bisulfite conversion reaction. For more information about EpiTect products, contact one of our Technical Service Departments (see back cover or visit www.qiagen.com).

Quantity of starting template

The annealing efficiency of a primer to the template is an important factor in PCR. Owing to the thermodynamic nature of the reaction, the primer:template ratio strongly influences the specificity and efficiency of PCR and should be optimized empirically. If too little template is used, primers may not be able to find their complementary sequences. Too much template may lead to an increase in mispriming events.

PCR optimization

The PyroMark PCR Kit will produce satisfactory results in most cases. However, if a higher Mg^{2+} concentration is required, we recommend using the 25 mM $MgCl_2$ provided in the kit.

The recommended annealing temperature is 60°C and 56°C for genomic DNA and bisulfite treated DNA, respectively, when using PyroMark ADSW 2.0.

Addition of Q-Solution® (provided with the PyroMark PCR Kit) can improve PCR yield and specificity for difficult templates that, for example, have a high degree of secondary structure or templates that are GC-rich.

For all PCR optimization tests, analyze 5 µl of a 25µl PCR on an agarose gel and aim for one strong specific band with minimal excess of primers.

Please refer to the *PyroMark PCR Handbook* for further troubleshooting.

Equal amplification of both alleles in AQ and CpG assays

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 the PyroMark Q24 MDx 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, including 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 a 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. An InDel heterozygote should give 50% deletion.

Sample preparation

Use 5–20 µl of a 25 µl PCR for immobilization to Streptavidin Sepharose High Performance (GE Healthcare) according to the instructions in Section 5.3.3. This will be equivalent to approximately 0.5–4 picomoles of PCR product, depending on the PCR efficiency.

Note: When using the PyroMark PCR Kit, 5–10 µl of the PCR product gives satisfactory Pyrosequencing results in most cases. This volume should be adjusted to achieve single peak heights of at least 40 RLU in the Pyrogram.

Pyrosequencing analysis

Use default settings in the software for all assay setups if not otherwise stated.

Assay setup

AQ and CpG assays

When creating an AQ or CpG assay, the sequence to analyze should contain a sufficient number of bases to generate at least five nonvariable reference peaks. For InDels, a few reference peaks should be included before the variable position.

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 and make suitable modifications when the icon appears.

Bisulfite treatment controls in CpG assays

When creating a CpG assay, inclusion of bisulfite treatment controls is recommended. Cytosines not followed by a Guanine, indicated as orange Ts in PyroMark ADSW, should be fully converted to Thymine during bisulfite treatment and can therefore be used as controls for the reaction. When creating a CpG assay in PyroMark Q24 MDx Software, the software indicates possible dispensations as controls for the bisulfite treatment reaction. The original sequence (before bisulfite treatment) can be entered in the assay and used to see if the suggested controls are Cs converted to **T**s (read as Gs and **A**s in a reverse assay) and suitable as controls, or not. The preferable controls are those dispensations that are located at the beginning of the sequence and/or represent single base incorporations.

SQA assays

Experience with sequencing large numbers of templates indicates that the dispensation order n(CTGA) gives, on average, the best sequencing quality. Individual templates may, however, give better results with other dispensation orders.

Where possible, SQA assays that involve resolution of different sequences should be designed such that resolution does not depend on accurate sequencing of homopolymers. In addition, it may be useful to have a few known bases at the beginning of the sequence, preferably single peaks. These can be used as reference peaks to aid the setting of the peak levels in difficult assays.

Ensure that the initial DNA sample is pure or that the assay is capable of specifically amplifying and/or sequencing only

one target sequence in the sample. The assay may otherwise generate mixed-sequence that cannot be analyzed.

Pay attention to tips and warnings indicated by the red icon and make suitable modifications when the icon appears.

Validation of a new assay

Controls

All new assays have to be validated by the user. Use a reference DNA sample when testing a new assay and ensure that appropriate analysis parameters in the PyroMark Q24 MDx 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:

- PCR without template DNA. This will show if the primers interact to give a background signal in Pyrosequencing reactions.
- 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.
- 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.
- Biotinylated primer without any PCR product. This will show if the biotinylated primer can form duplexes or hairpins and give background signal in Pyrosequencing reactions.
- Sequencing primer and biotinylated primer together without PCR product. This will show if the sequencing primer and the biotinylated primer can form duplexes and give background signal in Pyrosequencing reactions.

Pyrograms from these controls should not show any significant 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 ultimate goal for a well-optimized assay is that all variable positions in an AQ or CpG assay, or the sequence in the quality control window of a SQA assay, have the quality assessment "Passed" when using default or more stringent analysis parameters. Such results will be shown as blue in the 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

For samples and positive controls, aim for:

- Sufficient signal intensities. Aim for a single peak height of at least 40 RLU
- No background in blank dispensations
- No background in variable positions (AQ and CpG)
- Expected reference sequence pattern (AQ and CpG)
- All positions (AQ and CpG) and quality control window (SQA) with quality assessment "Passed".

The quality assessments for AQ and CpG assays are based on the sequence context as well as the results in the analyzed positions. Deviations from this built-in quality control are shown as warnings in the "Well Information" area.

The analysis results for SQA assays are based on the appearance of the peaks in Pyrogram, related to peak height levels estimated by the software.

The inclusion of known bases in SQA assays can improve the estimation of peak height level.

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Appendix C

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.

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