July 2009

PyroMark® Control Oligo Handbook

For use with PyroMark systems for installation check

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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QIAGEN sets standards in:

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

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Contents

Kit Contents

PyroMark Control Oligo Catalog no.	979203
Control Oligo 20 µM	50 µl
10x Dilution Buffer	2 x 1.7 ml
Handbook	1

Storage

The PyroMark Control Oligo should be stored at –20°C upon arrival. Repeated thawing and freezing (>5 x per year) should be avoided. The PyroMark Control Oligo is stable until the expiration date when stored under these conditions.

Product Use Limitations

PyroMark Control Oligo is intended for molecular biology applications. This product is neither intended for the diagnosis, prevention, or treatment of a disease, nor has it been validated for such use either alone or in combination with other products. Therefore, the performance characteristics of the products for clinical use (i.e., diagnostic, prognostic, therapeutic, or blood banking) are unknown.

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

Product Warranty and Satisfaction Guarantee

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

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

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

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

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

Quality Control

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

Safety Information

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

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

The PyroMark Control Oligo provides a means to verify proper installation of the PyroMark systems. In addition, the PyroMark Control Oligo can be used in troubleshooting to determine if an unexpected result is related to the instrument, to the PyroMark Vacuum Workstations, or to the assay.

Principle and procedure

The PyroMark Control Oligo is a biotinylated oligonucleotide, which allows the user to verify that all the PyroMark instruments and PyroMark Vacuum Workstations are functioning properly.

Under defined conditions, the oligonucleotide can form an internal stem–loop structure. This structure enables self-priming of the oligonucleotide for extension by the DNA polymerase and eliminates the need for a sequencing primer in the Pyrosequencing reaction. The sequenced region includes single bases of all nucleotides, homopolymers of 2 and 3 bases, and a wobbled/degenerated base. This variable position is automatically analyzed by the software, and results are presented as %C and %T. Figure 1 shows the structure of the oligonucleotide.

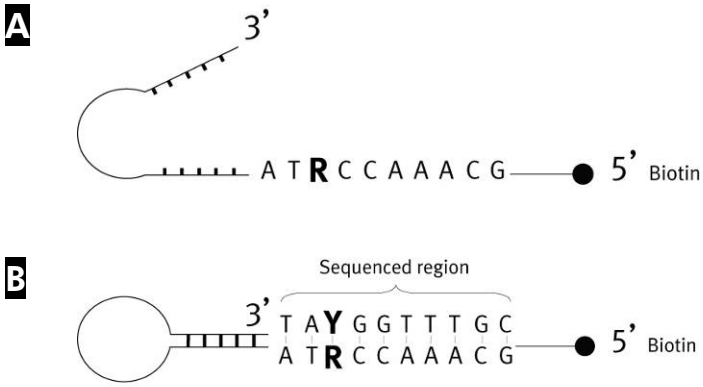

Figure 1. Structure of the PyroMark Control Oligo. A The open structure of the oligonucleotide. B The self-primed structure of the oligonucleotide, with the analyzed sequence indicated.

Description of protocols

It is recommended that 2 runs be performed to verify proper installation of the PyroMark instruments.

Function of the PyroMark Q24 Instrument

To verify correct function of the PyroMark Q24 Instrument, follow "Protocol: Verifying the Function of the PyroMark Q24 Instrument", page 10. The PyroMark Control Oligo is added directly to PyroMark Q24 Plate **without** prior preparation on the PyroMark Q24 Vacuum Workstation.

Function of the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation

To verify correct function of the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation, follow "Protocol: Verifying the Function of the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation", page 14. The PyroMark Control Oligo is prepared using the PyroMark Q24 Vacuum Workstation before analysis on the PyroMark Q24.

Troubleshooting the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation

To perform a troubleshooting of the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation, follow "Protocol: Troubleshooting Procedure for the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation", page 21. A Pyrosequencing reaction is performed with 8 wells containing the PyroMark Control Oligo and 8 wells containing the PyroMark Control Oligo prepared on the PyroMark Q24 Vacuum Workstation.

Troubleshooting the PyroMark Q96 ID Instrument and PyroMark Vacuum Workstation

To perform a troubleshooting of the PyroMark Q96 ID Instrument and PyroMark Q96 Vacuum Workstation, follow "Protocol: Troubleshooting Procedure for the PyroMark Q96 ID Instrument and PyroMark Q96 Vacuum Workstation", page 28. A Pyrosequencing reaction is performed with 8 wells containing the PyroMark Control Oligo and 8 wells containing the PyroMark Control Oligo prepared on the PyroMark Q96 Vacuum Workstation.

Troubleshooting the PyroMark Q96 MD Instrument and PyroMark Q96 Vacuum Workstation

To perform a troubleshooting of the PyroMark Q96 MD Instrument and PyroMark Q96 Vacuum Workstation, follow "Protocol: Troubleshooting Procedure for the Instrument and PyroMark Q96 Vacuum Workstation", page 36. A Pyrosequencing reaction is performed with 8 wells containing the PyroMark Control Oligo and 8 wells containing the PyroMark Control Oligo prepared on the PyroMark Q96 Vacuum Workstation.

Equipment and Reagents to Be Supplied by User

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

For use with the PyroMark Q24 System

- PyroMark Q24 Instrument (cat. no. 9001514)
- PyroMark Q24 Software (cat. no. 9019062)
- PyroMark Q24 Plate (100) (cat. no. 979201)
- PyroMark Q24 Cartridge (3) (cat. no. 979202)
- PyroMark Q24 Vacuum Workstation (cat. no. 9001518 [220V]; 9001516 [110V]; 9001519 [100V])*
- PyroMark Gold Q24 Reagents (5 x 24) (cat. no. 970802)

For use with the PyroMark Q96 ID System

- PyroMark Q96 ID Instrument (cat. no. 9001525)
- PyroMark Q96 ID Software (cat. no. 9019083)
- PyroMark Q96 Plate Low (100) (cat. no. 979002)
- PyroMark Q96 Cartridge (3) (cat. no. 979004)
- PyroMark Gold Q96 Reagents (5x96) (cat. no. 972804)
- PyroMark Q96 Vacuum Workstation (cat. no. 9001529 [220 V]; 9001528 [110V]; 9001740 [UK])

For use with the PyroMark Q96 MD System

- PyroMark Q96 MD Instrument (cat. no. 9001526)
- PyroMark Q96 MD Software (cat. no. 9019085)
- PyroMark Q96 HS Plate (100) (cat. no. 979101)
- PyroMark Q96 HS Dispensing Tip Holder (cat. no. 9019075)
- PyroMark Q96 HS Reagent Tips (4) (cat. no. 979102)
- PyroMark Q96 HS Nucleotide Tips (8) (cat. no. 979103)
- PyroMark Gold Q96 Reagents (5 x 96) (cat. no. 972804)
- PyroMark Q96 Vacuum Workstation (cat. no. 9001529 [220 V]; 9001528 [110V]; 9001740 [UK])

Additional equipment needed for all systems

- PyroMark Binding Buffer (cat. no. 979006)
- PyroMark Denaturation Solution (cat. no. 979007)

- PyroMark Wash Buffer, concentrate (cat. no. 979008)
- PyroMark Annealing Buffer (cat. no. 979009)
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80°C
- 24- or 96-well PCR plate or strips
- Strip caps
- 1.5 ml or 2 ml microcentrifuge tubes for dilution of the PyroMark Control Oligo
- Streptavidin Sepharose® High Performance (GE Healthcare, cat. no. 17-5113-01; www.gelifesciences.com)
- Pipets (adjustable)
- Sterile pipet tips
- High-purity water (Milli-Q® 18.2 MΩ x cm or equivalent)
- Ethanol (70%)

Protocol: Verifying the Function of the PyroMark Q24 Instrument

This protocol describes how to use the PyroMark Control Oligo to verify the function of PyroMark Q24 Instrument. To verify the function of the PyroMark Q24 Instrument and the PyroMark Q24 Vacuum Workstation, see "Protocol: Verifying the function of the PyroMark Q24 Instrument and the PyroMark Q24 Vacuum Workstation", page 14.

Important point before starting

- For further information on how to create an Assay Setup and a Run Setup, see the *PyroMark Q24 Software User Guide*.

Things to do before starting

- Follow the instructions in *PyroMark Q24 User Manual* to install the PyroMark Q24 Instrument.
- The dilution buffer provided with the PyroMark Control Oligo needs to be diluted before use. Prepare 1x dilution buffer by mixing 200 µl of 10x dilution buffer with 1800 µl of high-purity water.
- Place the PyroMark Q24 Plate Holder on a heating block at 80°C for use in step 11.
- Allow all required reagents and solutions to reach room temperature (15–25°C) before starting.

Procedure

1. **Set up an assay for the PyroMark Control Oligo by using the PyroMark Q24 Software.**
2. **Click in the toolbar and select "New AQ Assay".**
3. **Type the following sequence in "Sequence to Analyze".**
   TAYGGTTTGC

   For more information on how to create an Assay Setup file, see the *PyroMark Q24 Analysis Software User Guide*.
4. **Click the "Generate Dispensation Order" icon to get the following nucleotide dispensation order:**
   CTGACTGTG

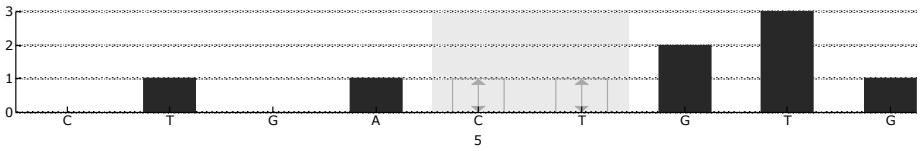

Figure 2. Histogram for AQ mode. Nucleotide additions 1 and 3 are blank dispensations and serve as negative controls. The fifth and the sixth dispensations analyze the variable position (wobbled/degenerated base).

5. **Click in the toolbar to save the assay.**
6. **Create a Run Setup by importing the assay parameters to all 24 wells.**

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

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

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

   For more information on how to create a Run Setup file, see the *PyroMark Q24 Software User Guide*.
7. **Save the Run Setup to a USB memory stick (supplied with the PyroMark Q24 system).**
8. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Select "Pre Run Information" from the "Tools" menu and, when the report appears, click .**
9. **Dilute the PyroMark Control Oligo to 0.04 µM as shown in Table 1.**

Table 1. Dilution of the PyroMark Control Oligo

Component	Volume	Concentration
PyroMark Control Oligo	10 µl	20 µM
1x Dilution buffer*	90 µl	–
First serial dilution	**100 µl**	**2 µM**
First serial dilution (from above)	30 µl	2 µM
1x Dilution buffer*	1470 µl	–
Final dilution	**1500 µl**	**0.04 µM**

* Make sure that the 10x dilution buffer supplied with the PyroMark Control Oligo is diluted with high-purity water before use. See "Things to do before starting", page 10.

10. **Add 25 µl of the diluted (0.04 µM) PyroMark Control Oligo to each well of a PyroMark Q24 Plate.**
11. **Heat the PyroMark Q24 Plate with the PyroMark Oligo at 80°C for 2 min using a heating block and the prewarmed PyroMark Q24 Plate Holder.**
12. **Remove the PyroMark Q24 Plate from the plate holder, and let the samples cool to room temperature (15–25°C) for at least 5 min.**
13. **Load the PyroMark Q24 Cartridge with the appropriate volumes of PyroMark Gold Q24 Reagents, as given in the Pre Run Information report from step 8.**

    The Pre Run Information report, found in the "Tools" menu at run setup (see the *PyroMark Q24 Software User Guide*), provides information about the volume of nucleotides, enzyme mixture, and substrate mixture needed for the assay.
14. **Open the cartridge gate and insert the filled PyroMark Q24 Cartridge with the label facing out. Push the cartridge in fully and then push it down.**
15. **Ensure that the cartridge is properly inserted and close the gate.** Refer to the *PyroMark Q24 User Manual* for more information.
16. **Open the plate-holding frame and place the plate on the heating block.**
17. **Close the plate-holding frame and the instrument lid.**
18. **Insert the USB memory stick (containing the run file) into the USB port at the front of the instrument.**

    Do not remove the USB memory stick before the run is finished.
19. **Select "Run" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".**
20. **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".
21. **When the run file is selected, press "Select" to start the run.**
22. **When the run is finished and the instrument confirms that the run file has been saved to the USB memory stick, press "Close".**
23. **Remove the USB memory stick.**
24. **Open the instrument lid.**
25. **Open the cartridge gate and remove the PyroMark Q24 Cartridge by lifting it up and pulling it out.**
26. **Close the gate.**
27. **Open the plate-holding frame and remove the PyroMark Q24 Plate from the heating block.**

28. **Close the plate-holding frame and the instrument lid.**
29. **Discard the PyroMark Q24 Plate and clean the PyroMark Q24 Cartridge (see the *PyroMark Gold Q24 Reagents Handbook*).**
30. **Open the run in the PyroMark Q24 Software and analyze all wells. The peak pattern for Run 1 should look like the one in Figure 3.**

    To obtain peak height values, select "Export Peak Heights" from the "Tools" menu. Save the data in a suitable format (*.csv or *.tsv). Open this file in Microsoft® Excel (Delimited), and calculate the mean single peak height for each well as described on the next page.

- **Perform a quality assessment.**
  All wells should give "Passed" quality, shown as a blue bar in the bottom field of the well when looking at the overview tab and with %C indicated in a blue rectangle in the Pyrogram. If the quality assessment is "Check" or "Failed", look in "Well Information" for explanations.
- **Evaluate peak heights.**
  The single peak height (dispensations 2, 4, and 9) should approximately be 50 RLU.

If the values are within the set limits, the system is properly installed. If the results are not as stated above, see "Troubleshooting Guide", page 44, for possible reasons for the failure and rerun Run 1. If the repeat of Run 1 fails, 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).

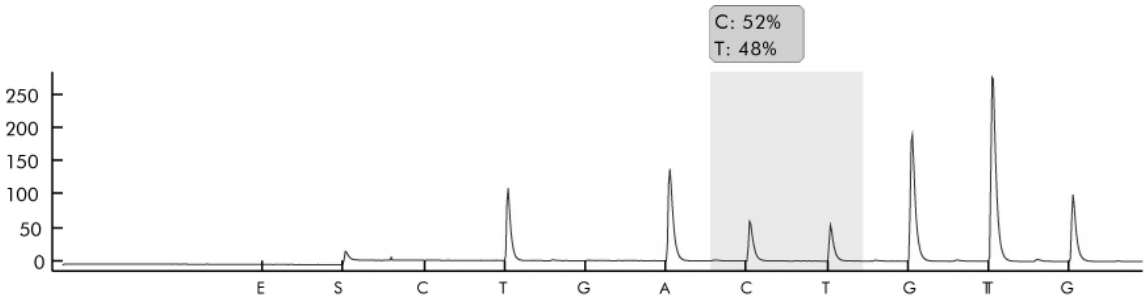

Figure 3. Pyrogram of Run 1.

Protocol: Verifying the Function of the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation

This protocol describes how to use the PyroMark Control Oligo to verify the function of the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation. To verify the function of the PyroMark Q24 Instrument only, see "Protocol: Verifying the Function of the PyroMark Q24 Instrument", page 10.

Important point before starting

- For further information on how to create an Assay Setup and a Run Setup, see the *PyroMark Q24 Software User Guide*.

Things to do before starting

- Follow the instructions in *PyroMark Q24 User Manual* to install the PyroMark Q24.
- The dilution buffer provided with the PyroMark Control Oligo needs to be diluted before use. Prepare 1x dilution buffer by mixing 200 µl of 10x dilution buffer with 1800 µl of high-purity water.
- Place the PyroMark Q24 Plate Holder on a heating block at 80°C for use in step 30.
- Allow all required reagents and solutions to reach room temperature (15–25°C) before starting.

Procedure

1. **Set up an assay for the PyroMark Control Oligo by using the PyroMark Q24 Software.**
2. **Click in the toolbar and select "New AQ Assay".**
3. **Type the following sequence in "Sequence to Analyze".**
   TAYGGTTTGCA

   For more information on how to create an Assay Setup file, see the *PyroMark Q24 Software User Guide*.
4. **Manually enter the following "Dispensation Order".**
   ACGTTATCGTTGC

   For more information on how to create an Assay Setup file, see the *PyroMark Q24 Software User Guide*.

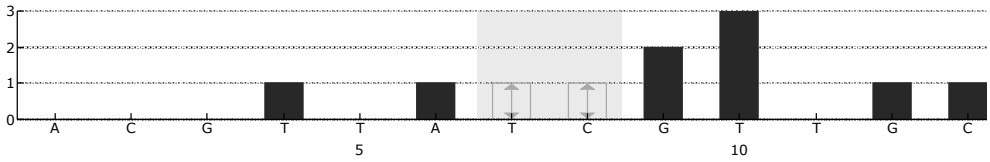

Figure 4. Histogram for AQ mode. Nucleotide additions 1, 2, 3, 5, and 11 are blank and serve as negative controls. The seventh and the eight dispensations analyze the variable position.

5. **Click in the toolbar to save the assay.**
6. **Create a Run Setup by importing the assay parameters to all 24 wells.**

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

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

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

   For more information on how to create a Run Setup file, see the *PyroMark Q24 Software User Guide*.
7. **Save the Run Setup to a USB memory stick (supplied with the PyroMark Q24 system).**
8. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Select "Pre Run Information" from the "Tools" menu and, when the report appears, click .**
9. **Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.**
10. **Prepare a master mix for DNA immobilization according to Table 2. Prepare a volume 10% greater than that required for the total number of reactions to be performed.**

Table 2. Master mix for DNA immobilization

Number of samples	**1**	**26***
Streptavidin Sepharose High Performance	2 µl	52 µl
PyroMark Binding Buffer	40 µl	1040 µl
High-purity water	13 µl	338 µl
Total volume	**55 µl**	**1430 µl**

* Provides a sufficient amount for the 24 samples required.

11. Dilute the PyroMark Control Oligo to 0.04 µM as shown in Table 3.

Table 3. Dilution of the PyroMark Control Oligo

Component	Volume	Concentration
PyroMark Control Oligo	10 µl	20 µM
1x Dilution buffer*	90 µl	–
First serial dilution	**100 µl**	**2 µM**
First serial dilution (from above)	30 µl	2 µM
1x Dilution buffer*	1470 µl	–
Final dilution	**1500 µl**	**0.04 µM**

* Make sure that the 10x dilution buffer supplied with the PyroMark Control Oligo is diluted with high-purity water before use. See Things to do before starting", page 14.

12. Shake the tube containing the master mix, and add 55 µl of the master mix and 25 µl of the diluted (0.04 µM) PyroMark Control Oligo to all 24 wells of a 24-well PCR plate or strips.

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

14. Agitate the PCR plate at room temperature (15–25°C) for 5–10 min at 1400 rpm.

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

During this step, prepare the PyroMark Q24 Vacuum Workstation for sample preparation (see Appendix A, page 53).

15. Add 25 µl of PyroMark Annealing Buffer to each well of a PyroMark Q24 Plate.

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

16. Place the PCR plate (or strips) and the PyroMark Q24 Plate on the worktable of the PyroMark Q24 Vacuum Workstation (see Figure 5).

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

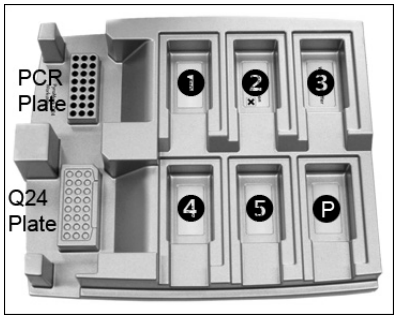

Figure 5. Placement of PCR plate (or strips) and PyroMark Q24 Plate on the PyroMark Q24 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4**, **5**). **P**: Parking position.

17. **Apply vacuum to the vacuum tool by opening the vacuum switch.**
18. **Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool.**

    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.
19. **Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.**
20. **Transfer the tool to the trough containing PyroMark Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
21. **Transfer the tool to the trough containing PyroMark Wash Buffer (trough 3). Flush the filter probes for 10 s.**
22. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (see Figure 6).**

Figure 6. Illustration of the vacuum tool raised to beyond 90° vertical.

23. **While the tool is held over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).**

24. **Release the beads in the plate containing 25 µl PyroMark Annealing Buffer by shaking the tool from side to side. Allow the filter probes to rest on the bottom of the wells.**
25. **Transfer the tool to the first trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
26. **Wash the filter probes by lowering the probes into the second trough with high-purity water (trough 5) and applying vacuum. Flush the probes with 70 ml high-purity water.**
27. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (see Figure 6).**
28. **Close the vacuum switch on the tool (Off), and place the tool in the Parking (P) position.**
29. **Turn off the vacuum pump.**

    At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage, see Appendix C, page 55.
30. **Heat the PyroMark Q24 Plate with the samples at 80°C for 2 min using a heating block and the prewarmed PyroMark Q24 Plate Holder.**
31. **Remove the PyroMark Q24 Plate from the plate holder, and let the samples cool to room temperature (15–25°C) for at least 5 min.**
32. **Load the PyroMark Q24 Cartridge with the appropriate volumes of PyroMark Gold Q24 Reagents, as given in the Pre Run Information report from step 8.**

    The Pre Run Information report, found in the "Tools" menu at run setup (see the *PyroMark Q24 Software User Guide*), provides information about the volume of nucleotides, enzyme mixture, and substrate mixture needed for the assay.
33. **Open the cartridge gate and insert the filled PyroMark Q24 Cartridge with the label facing out. Push the cartridge in fully and then push it down.**
34. **Ensure the cartridge is properly inserted and close the gate.**
    Refer to the *PyroMark Q24 User Manual* for more information.
35. **Open the plate-holding frame and place the plate on the heating block.**
36. **Close the plate-holding frame and the instrument lid.**
37. **Insert the USB memory stick (containing the run file) into the USB port at the front of the instrument.**

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

39. **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".

40. **When the run file is selected, press "Select" to start the run.**
41. **When the run is finished and the instrument confirms that the run file has been saved to the USB memory stick, press "Close".**
42. **Remove the USB memory stick.**
43. **Open the instrument lid.**
44. **Open the cartridge gate and remove the PyroMark Q24 Cartridge by lifting it up and pulling it out.**
45. **Close the gate.**
46. **Open the plate-holding frame and remove the PyroMark Q24 Plate from the heating block.**
47. **Close the plate-holding frame and the instrument lid.**
48. **Discard the PyroMark Q24 Plate and clean the PyroMark Q24 Cartridge (see the *PyroMark Gold Q24 Reagents Handbook*).**
49. **Open the run in the PyroMark Q24 Software and analyze all wells. The peak pattern for Run 2 should look like the one in Figure 7.**

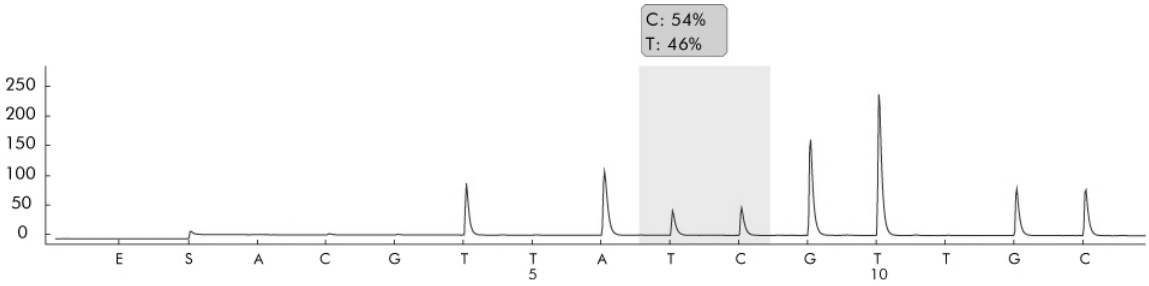

Figure 7. Pyrogram of Run 2.

50. **Confirm the proper installation of the system and use of the reagents by evaluating the quality assessment, quantification results, single peak heights, and background.**

    To obtain peak height values, select "Export Peak Heights" from the "Tools" menu. Save the data in a suitable format (*.csv or *.tsv). Open this file in Microsoft Excel (Delimited), and calculate the mean single peak height and background for each well as described below.

    - **Perform a quality assessment.**
      All wells should give "Passed" quality, shown as a blue bar in the bottom field of the well when looking at the overview tab and with % C indicated in a blue rectangle in the Pyrogram. If the quality assessment is "Check" or "Failed", look in "Well Information" for explanations.
    - **Evaluate the quantification results.**
      Select the "AQ Analysis Statistics Report" from the "Reports" menu. Quantification results are given in the report with standard deviation.

The %C should be in the range 40–60%. The standard deviation should not exceed 2% units.

- **Evaluate single peak heights.**
  The mean single peak height should ideally be 50 ±15 RLU.

$$\text{Mean single peak height} = \frac{\text{Sum single peaks (dispensation 4, 6, 12, 13)}}{4}$$

- **Evaluate the background.**
  Background from blank dispensations should not exceed 3%.

$$\text{Background (\%)} = \frac{\text{Sum blanks (dispensation 1, 2, 3, 5)}}{\text{Sum single peaks (dispensation 4, 6, 12, 13)}} \times 100$$

If the values are within the set limits, the system is properly installed. If the results are not as stated above, see "Troubleshooting Guide", page 44, for possible causes and actions to be taken. If the troubleshooting guide does not explain the problem, 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).

Protocol: Troubleshooting Procedure for the PyroMark Q24 Instrument and PyroMark Q24 Vacuum Workstation

If an unexpected result has been obtained it is crucial to determine whether this is related to the PyroMark Q24 Instrument, the PyroMark Q24 Vacuum Workstation, or the assay. This protocol describes how to use the PyroMark Control Oligo to verify the function of the PyroMark Q24 Instrument, comparing results with or without the PyroMark Q24 Vacuum Workstation.

Important point before starting

- For further information on how to create an Assay Setup and a Run Setup, see the *PyroMark Q24 Software User Guide*.

Things to do before starting

- Follow the instructions in *PyroMark Q24 User Manual* to install the PyroMark Q24.
- The dilution buffer provided with the PyroMark Control Oligo needs to be diluted before use. Prepare 1x dilution buffer by mixing 200 µl of 10x dilution buffer with 1800 µl of high-purity water.
- Place the PyroMark Q24 Plate Holder on a heating block at 80°C for use in step 30.
- Allow all required reagents and solutions to reach room temperature (15–25°C) before starting.

Procedure

1. **Set up an assay for the PyroMark Control Oligo by using the PyroMark Q24 Software.**
2. **Click in the toolbar and select "New AQ Assay".**
3. **Type the following sequence in "Sequence to Analyze".**
   TAYGGTTTGCA

   For more information on how to create an Assay Setup file, see the *PyroMark Q24 Software User Guide*.
4. **Manually enter the following "Dispensation Order".**
   ACGTTATCGTTGC

   For more information on how to create an Assay Setup file, see the *PyroMark Q24 Software User Guide*.

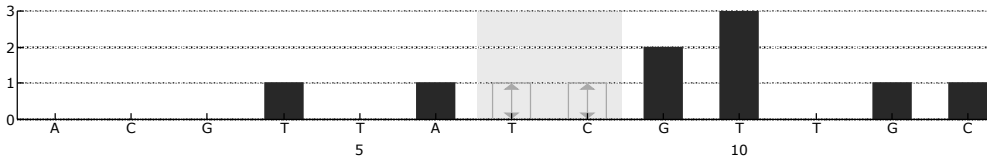

Figure 8. Histogram for AQ mode. Nucleotide additions 1, 2, 3, 5, and 11 are blank and serve as negative controls. The seventh and the eight dispensations analyze the variable position.

5. **Click in the toolbar to save the assay.**
6. **Create a Run Setup by importing the assay parameters to the appropriate wells.**

   We recommend using 16 wells: 8 wells for samples prepared using the PyroMark Q24 Vacuum Workstation and 8 samples added directly to PyroMark Q24 Plate.

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

   - Right-click the well and select "Load Assay" from the context menu
   - Select the assay in the shortcut browser, and click and drag the assay to the well.
   - Recommended: Fill in Sample ID, Plate ID, Barcode, Reagent ID, and Run Note.

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

   For more information on how to create a Run Setup file, see the *PyroMark Q24 Software User Guide*.

7. **Save the Run Setup to a USB memory stick (supplied with the PyroMark Q24 system).**
8. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Select "Pre Run Information" from the "Tools" menu and, when the report appears, click .**
9. **Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.**
10. **Prepare a master mix for DNA immobilization according to Table 4. Prepare a volume 10% greater than that required for the total number of reactions to be performed.**

Table 4. Master mix for DNA immobilization

Number of samples	1	9*
Streptavidin Sepharose High Performance	2 µl	18 µl
PyroMark Binding Buffer	40 µl	360 µl
High-purity water	13 µl	117 µl
Total volume	**55 µl**	**495 µl**

* Provides a sufficient amount for the 8 samples required.

11. Dilute the PyroMark Control Oligo to 0.04 µM as shown in Table 5.

Table 5. Dilution of the PyroMark Control Oligo

Component	Volume	Concentration
PyroMark Control Oligo	10 µl	20 µM
1x Dilution buffer*	90 µl	–
First serial dilution	**100 µl**	**2 µM**
First serial dilution (from above)	30 µl	2 µM
1x Dilution buffer*	1470 µl	–
Final dilution	**1500 µl**	**0.04 µM**

* Make sure that the 10x dilution buffer supplied with the PyroMark Control Oligo is diluted with high-purity water before use. See "Things to do before starting", page 21.

12. Shake the tube containing the master mix, and add 55 µl of the master mix and 25 µl of the diluted (0.04 µM) PyroMark Control Oligo to 8 wells of a 24-well PCR plate or strips.

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

14. Agitate the PCR plate at room temperature (15–25°C) for 5–10 min at 1400 rpm.

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

During this step, prepare the PyroMark Q24 Vacuum Workstation for sample preparation (see Appendix A, page 53).

15. **Add 25 µl of PyroMark Annealing Buffer to each well of the PyroMark Q24 Plate that will be used with the immobilized PyroMark Control Oligo to be processed with the PyroMark Q24 Vacuum Workstation. Add 25 µl of the diluted (0.04 µM) PyroMark Q24 Control Oligo to 8 additional wells according to the Run Setup.**

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

16. **Place the PCR plate (or strips) and the PyroMark Q24 Plate on the worktable of the PyroMark Q24 Vacuum Workstation (see Figure 9).**

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

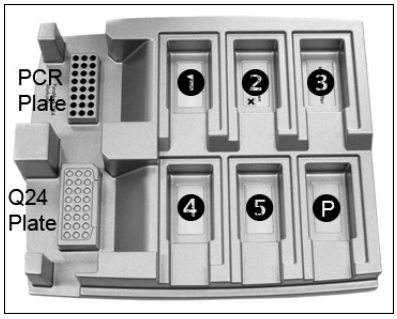

Figure 9. Placement of PCR plate and PyroMark Q24 Plate on the PyroMark Q24 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4**, **5**). **P**: Parking position.

17. **Apply vacuum to the tool by opening the vacuum switch.**
18. **Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool.**

    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.

19. **Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.**
20. **Transfer the tool to the trough containing PyroMark Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
21. **Transfer the tool to the trough containing PyroMark Wash Buffer (trough 3). Flush the filter probes for 10 s.**
22. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (see Figure 10).**

Figure 10. Illustration of the vacuum tool raised to beyond 90° vertical.

23. **While the tool is held over the PyroMark Q24 Plate, close the vacuum switch on the tool (Off).**
24. **Release the beads in the plate containing 25 µl PyroMark Annealing Buffer by shaking the tool from side to side. Allow the filter probes to rest on the bottom of the wells.**
25. **Transfer the tool to the first trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
26. **Wash the filter probes by lowering the probes into the second trough with high-purity water (trough 5) and applying vacuum. Flush the probes with 70 ml high-purity water.**
27. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (see Figure 10).**
28. **Close the vacuum switch on the tool (Off), and place the tool in the Parking (P) position.**
29. **Turn off the vacuum pump.**

    At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q24 Vacuum Workstation should be checked for dust and spillage, see Appendix C, page 55.
30. **Heat the PyroMark Q24 Plate with the samples at 80°C for 2 min using a heating block and the prewarmed PyroMark Q24 Plate Holder.**
31. **Remove the PyroMark Q24 Plate from the plate holder, and let the samples cool to room temperature (15–25°C) for at least 5 min.**
32. **Load the PyroMark Q24 Cartridge with the appropriate volumes of PyroMark Gold Q24 Reagents, as given in the Pre Run Information report from step 8.**

    The Pre Run Information report, found in the "Tools" menu at run setup (see the *PyroMark Q24 Software User Guide*), provides information about the volume of nucleotides, enzyme mixture, and substrate mixture needed for the assay.

33. **Open the cartridge gate and insert the filled PyroMark Q24 Cartridge with the label facing out. Push the cartridge in fully and then push it down.**
34. **Ensure the cartridge is properly inserted and close the gate.**
    Refer to the *PyroMark Q24 User Manual* for more information.
35. **Open the plate-holding frame and place the plate on the heating block.**
36. **Close the plate-holding frame and the instrument lid.**
37. **Insert the USB memory stick (containing the run file) into the USB port at the front of the instrument.**
    Do not remove the USB memory stick before the run is finished.
38. **Select "Run" in the main menu (using the ▲ and ▼ screen buttons) and press "OK".**
39. **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".
40. **When the run file is selected, press "Select" to start the run.**
41. **When the run is finished and the instrument confirms that the run file has been saved to the USB memory stick, press "Close".**
42. **Remove the USB memory stick.**
43. **Open the instrument lid.**
44. **Open the cartridge gate and remove the PyroMark Q24 Cartridge by lifting it up and pulling it out.**
45. **Close the gate.**
46. **Open the plate-holding frame and remove the PyroMark Q24 Plate from the heating block.**
47. **Close the plate-holding frame and the instrument lid.**
48. **Discard the PyroMark Q24 Plate and clean the PyroMark Q24 Cartridge (see the *PyroMark Gold Q24 Reagents Handbook*).**
49. **Open the run in the PyroMark Q24 Software and analyze all wells. The peak pattern should look like the one in Figure 11.**

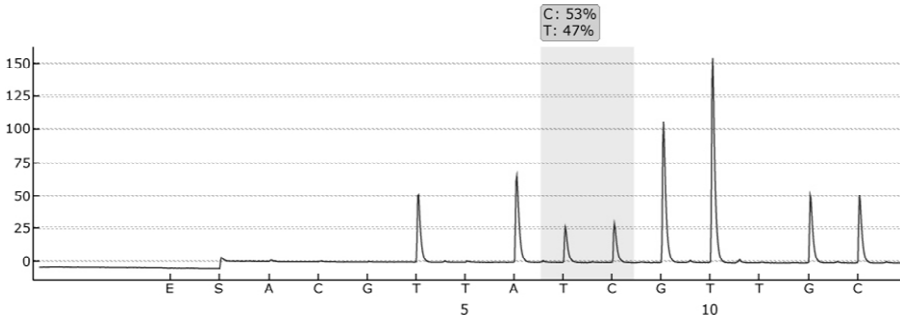

Figure 11. Pyrogram of PyroMark Q24 Troubleshooting Run.

50. Confirm the proper installation of the system and use of the reagents by evaluating the quality assessment, quantification results, single peak heights, and background.

To obtain peak height values, select "Export Peak Heights" from the "Tools" menu. Save the data in a suitable format (*.csv or *.tsv). Open this file in Microsoft Excel (Delimited), and calculate the mean single peak height and background for each well as described below.

- **Perform a quality assessment.**
  All wells should give "Passed" quality, shown as a blue bar in the bottom field of the well when looking at the overview tab and with %C indicated in a blue rectangle in the Pyrogram. If the quality assessment is "Check" or "Failed", look in "Well Information" for explanations.
- **Evaluate the quantification results.**
  Select the "AQ Analysis Statistics Report" from the "Reports" menu. Quantification results are given in the report with standard deviation. The %C should be in the range 40–60%. The standard deviation should not exceed 2 percentage units.
- **Evaluate single peak heights.**
  The mean single peak height should ideally be 50 ± 15 RLU.

$$\text{Mean single peak height} = \frac{\text{Sum single peaks (dispensation 4, 6, 12, 13)}}{4}$$

- **Evaluate the background.**
  Background from blank dispensations should not exceed 3%.

$$\text{Background (\%)} = \frac{\text{Sum blanks (dispensation 1, 2, 3, 5)}}{\text{Sum single peaks (dispensation 4, 6, 12, 13)}} \times 100$$

51. Evaluate the difference in peak heights with and without sample preparation. The reduction in peak height between samples prepared using the PyroMark Q24 Vacuum Workstation compared with PyroMark Control Oligo added directly to the PyroMark Q24 Plate should not be more than 20%.

If the values are within the set limits, the system is properly installed. If the results are not as stated above, see "Troubleshooting Guide", page 44, for possible causes and actions to be taken. If the troubleshooting guide does not explain the problem, 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).

Protocol: Troubleshooting Procedure for the PyroMark Q96 ID Instrument and PyroMark Q96 Vacuum Workstation

If an unexpected result has been obtained, it is crucial to determine whether this is related to the PyroMark Q96 ID Instrument, the PyroMark Q96 Vacuum Workstation, or the assay. This protocol describes how to use the PyroMark Control Oligo to verify the function of the PyroMark Q96 ID Instrument and to compare results of samples prepared with or without the PyroMark Q96 Vacuum Workstation.

Important point before starting

- Assay and run files can be set up using either PyroMark Q96 ID Software or PyroMark CpG Software
- For further information on how to create an Assay Setup and a Run Setup, see the *PyroMark Q96 ID Software Online Help*.
- For further information on how to create an Assay Setup and a Run Setup with the PyroMark CpG Software see the *PyroMark CpG Software Online Help*.

Things to do before starting

- Follow the instructions in *PyroMark Q96 ID User Manual* to install the PyroMark Q96 ID.
- The dilution buffer provided with the PyroMark Control Oligo needs to be diluted before use. Prepare 1x dilution buffer by mixing 200 µl of 10x dilution buffer with 1800 µl of high-purity water.
- Place the PyroMark Q96 Sample Prep Thermoplate Low on a heating block at 80°C for use in step 30.
- Allow all required reagents and solutions to reach room temperature (15–25°C before starting.

Procedure

1. **Set up a simplex entry for the PyroMark Control Oligo by using the PyroMark Q96 ID Software alternatively an assay in the PyroMark CpG Software using the parameters below.**
2. **Type the following sequence in "Sequence to Analyze".**
   TAYGGTTTGCA
3. **Manually enter the following "Dispensation Order".**
   ACGTTATCGTTGC

For more information on how to create an Assay Setup file, see the *PyroMark Q96 ID Software Online Help* or the *PyroMark CpG Software Online Help*.

Note: For the CpG assay, ignore the warning about sequence direction that is shown when the sequence to analyze and dispensation order is added.

Note: For a thorough control of the instrument, sample preparation and reagents, additional blanks are included in the dispensing order.

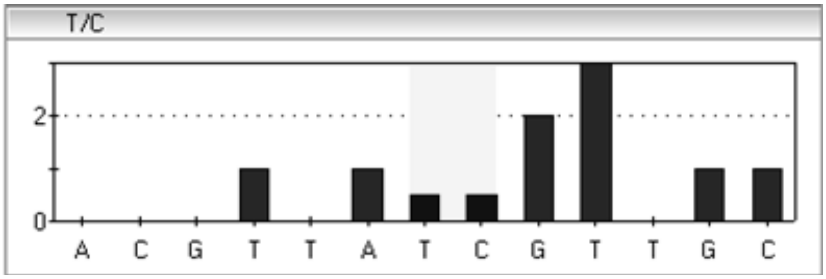

Figure 12. Histogram from PyroMark Q96 ID Software. Nucleotide additions 1, 2, 3, 5 and 11 are blank dispensations and serve as negative controls. The seventh and the eighth dispensations analyze the variable position (wobbled/degenerated base).

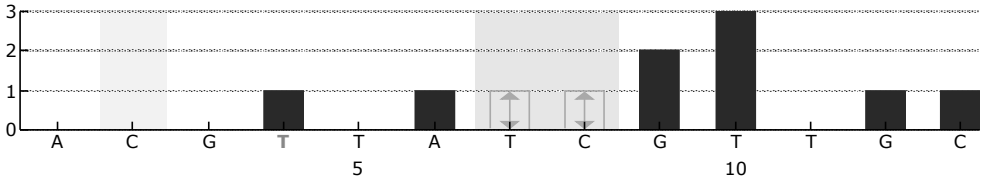

Figure 13. Histogram from PyroMark CpG Software. Nucleotide additions 1, 2, 3, 5 and 11 are blank dispensations and serve as negative controls. The seventh and the eighth dispensations analyze the variable position (wobbled/degenerated base).

4. **Save the entry (PyroMark Q96ID Software)/assay (PyroMark CpG Software).**
5. **Open a new run and allocate the created entry/assay to appropriate wells. Choose instrument parameters supplied by QIAGEN according to the reagents and cartridge that will be used for the run and save the run.**

We recommend using 16 wells: 8 wells for samples prepared using the PyroMark Q96 Vacuum Workstation and 8 samples added directly to PyroMark Q96 Plate Low.

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

For more information on how to create a Run Setup file in PyroMark Q96 ID Software, see the *PyroMark Q96 ID Software Online Help*.

For more information on how to create a Run Setup file in PyroMark CpG Software, see the *PyroMark CpG Software Online Help*.

6. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup.**

   Note: To generate a list of required volumes in the PyroMark Q96 ID Software click **View,** and **Run** in the browser area.

   To generate a list of required volumes in the PyroMark CpG Software, open the run file and select **Volume Information** from the **Tools** menu.

7. **Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.**
8. **Prepare a master mix for DNA immobilization according to Table 6. Prepare a volume 10% greater than that required for the total number of reactions to be performed.**

   Note: Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.

Table 6. Master mix for DNA immobilization

Component	Volume/sample
Streptavidin Sepharose High Performance	3 µl
PyroMark Binding Buffer	37 µl
Total volume	**40 µl**

9. **Dilute the PyroMark Control Oligo to 0.05 µM as shown in Table 7.**

Table 7. Dilution of the PyroMark Control Oligo

Component	Volume	Concentration
PyroMark Control Oligo	10 µl	20 µM
1x Dilution buffer*	90 µl	–
First serial dilution	**100 µl**	**2 µM**
First serial dilution (from above)	20 µl	2 µM
1x Dilution buffer*	780 µl	–
Final dilution	**800 µl**	**0.05 µM**

* Make sure that the 10x dilution buffer supplied with the PyroMark Control Oligo is diluted with high-purity water before use. See "Things to do before starting", page 28.

10. **Add 40 µl of the master mix and 40 µl of the diluted (0.05 µM) PyroMark Control Oligo to 8 wells of a 96-well PCR plate or strips.**
11. **Seal the PCR plate (or strips) using strip caps.**
12. **Agitate the PCR plate at room temperature (15–25°C) for 5–10 min at 1400 rpm.**

    During this step, prepare the PyroMark Q96 Vacuum Workstation for sample preparation (see Appendix B, page 54).

    Note: Sepharose beads sediment quickly. Capturing of beads must take place immediately following agitation.
13. **Add 40 µl of PyroMark Annealing Buffer to the wells of the PyroMark Q96 Plate Low that will be used with the immobilized PyroMark Control Oligo to be processed with the PyroMark Q96 Vacuum Workstation. Add 40 µl of the diluted (0.05 µM) PyroMark Control Oligo to 8 additional wells according to the Run Setup.**

    Since the oligonucleotide is self-primed, no sequencing primer is required. The beads are released into PyroMark Annealing Buffer.
14. **Place the PCR plate (or strips) and the PyroMark Q96 Plate Low on the worktable of the PyroMark Q96 Vacuum Workstation (see Figure 14).**

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

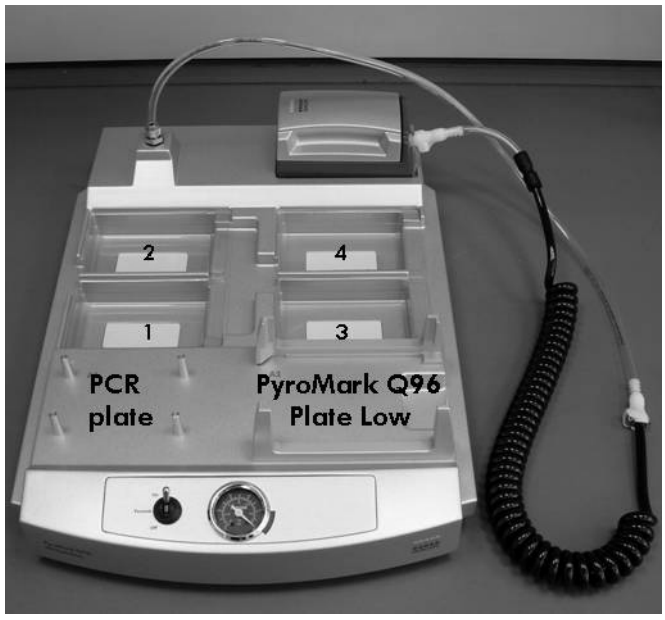

Figure 14. Placement of PCR plate (or strips) and PyroMark Q96 Plate Low on the PyroMark Q96 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4**).

15. **Apply vacuum to the tool by opening the vacuum switch on the vacuum workstation.**

16. **Wash the filter probes by lowering the probes into high-purity water (parking position). Let approximately 180 ml of water flush through the filter probes; i.e. empty the trough.**
17. **Refill the parking position with 180 ml high-purity water.**
18. **Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool.**

    Note: Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.
19. **Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.**
20. **Transfer the tool to the trough containing PyroMark Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
21. **Transfer the tool to the trough containing PyroMark Wash Buffer (trough 3). Flush the filter probes for 10 s.**
22. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes.**

Figure 15. Illustration of the vacuum tool raised to beyond 90° vertical.

23. **While the tool is held over the PyroMark Q96 Plate Low, close the vacuum switch on the vacuum workstation (Off).**
24. **Release the beads in the plate containing 40 µl PyroMark Annealing Buffer by shaking the tool from side to side. Allow the filter probes to rest on the bottom of the wells.**
25. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**

26. **Wash the filter probes by lowering the probes into the second trough with high-purity water (parking position) and applying vacuum. Flush the probes with 180 ml high-purity water.**
27. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (see Figure 15).**
28. **Turn off the vacuum switch on the vacuum workstation (Off), and place the tool in the Parking (P) position.**
29. **Turn off the vacuum pump.**

    At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q96 Vacuum Workstation should be checked for dust and spillage, see Appendix C, page 55.
30. **Heat the PyroMark Q96 Plate Low with the samples at 80°C for 2 min using a heating block and the prewarmed PyroMark Q96 Sample Prep Thermoplate Low.**
31. **Remove the PyroMark Q96 Plate from the thermo plate, and let the samples cool to room temperature (15–25°C) for at least 5 min.**
32. **Load the PyroMark Q96 Cartridge with the appropriate volumes of PyroMark Gold Q96 Reagents.**
    Note: To generate a list of required volumes in the PyroMark Q96 ID Software click **View,** and **Run** in the browser area.
    To generate a list of required volumes in the PyroMark CpG Software, open the run file and select **Volume Information** from the **Tools** menu.
33. **Switch on the instrument.**
34. **Open the instrument lid.**
35. **Open the process chamber lid.**
36. **Open the plate-holding frame.**
37. **Place the PyroMark Q96 Plate Low on the heating block.**
38. **Close the plate-holding frame and the process chamber lid.**
39. **Open the dispensing unit cover; release the latch, then open the cover.**
40. **Insert the filled dispensing cartridge with the label facing out.**
41. **Close the dispensing unit cover. Make sure that the latch snaps into its locked position.**
42. **Close the instrument lid.**
43. **Perform a run (see *PyroMark Q96 ID User Manual* or the *PyroMark CpG Software Online Help*).**
44. **After run open the instrument lid.**
45. **Open the dispensing unit and remove the reagent cartridge by lifting it up and pulling it out.**
46. **Close the dispensing unit.**

47. **Open the process chamber lid and remove the PyroMark Q96 Plate Low from the heating block (see *PyroMark Q96 ID User Manual*).**
48. **Close the process chamber and the instrument lid (see *PyroMark Q96 ID User Manual*).**
49. **Discard the PyroMark Q96 Plate Low and clean the PyroMark Q96 Cartridge (see the *PyroMark Gold Q96 Reagents Handbook*).**
50. **Open the run in the PyroMark Q96 ID Software/PyroMark CpG Software and analyze all wells (see *PyroMark Q96 ID Software Online Help/ PyroMark CpG Software Online help*). The peak pattern should look like the one in Figure 16.**

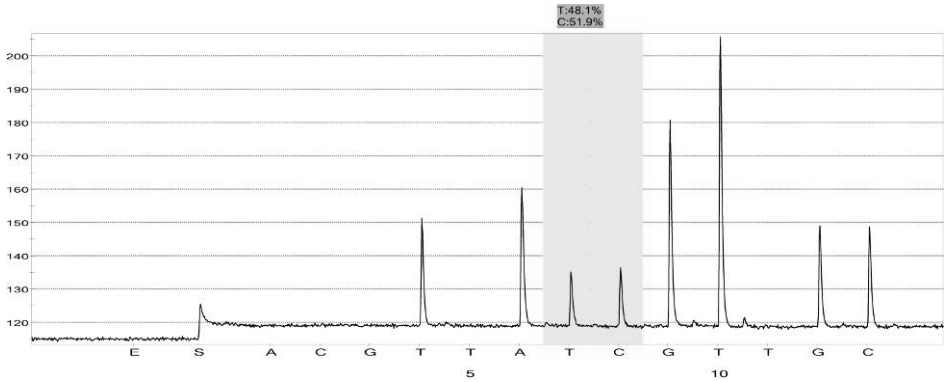

Figure 16. Pyrogram of the PyroMark Q96 ID Troubleshooting Run.

51. **Confirm the proper installation of the system and use of the reagents by evaluating the quality assessment, quantification results, background and difference in peak height with and without sample preparation.**

PyroMark Q96 ID Software: Select all wells by holding down the Ctrl or Shift key and click in the wells to mark them. Selected wells appear with a square around them. Click the arrow next to the **Peak Heights Button** on the **Peak Heights Tab.** Select **Export** from the drop-down list and save the data. Open this file in Microsoft Excel (Delimited) and calculate the mean single peak height and background for each well see below).

PyroMark CpG Software: Select **Peak Heights** from the **Reports** menu. Save the data in a suitable format (.csv or .tsv). Open this file in Microsoft Excel (Delimited) and calculate the mean single peak height and background for each well (see below).

Perform a quality assessment.
All wells should give "Passed" quality. If the quality assessment is "Check" or "Failed", look in "Well info" (PyroMark Q96 ID Software)/"Well Information" (PyroMark CpG Software) for explanations.

Evaluate the quantification results.
PyroMark Q96 ID Software: Quantification results with standard deviation are shown in **AQ Statistics** under the **Statistics Tab**. Select all wells by holding down the Ctrl or Shift key and click in the wells one-by-one to mark

them. Selected wells appear with a square around them. Click the Statistics button under the Statistics Tab.

PyroMark CpG Software: Quantification results with standard deviation are shown in the **CpG Statistics Report** that can be selected from the **Reports** menu.
The %C should be in the range 40 to 60 %. The standard deviation should not exceed 2% units.

Evaluate single peak heights.
The mean single peak height should ideally be 35 ± 10 RLU.

Mean single peak height =	Sum single peaks (dispensation 4, 6, 12, 13)
	4

Evaluate the background.
Background from blank dispensations should not exceed 5%.

Background (%) =	Sum blanks (dispensation 1, 2, 3, 5)	x 100
	Sum single peaks (dispensation 4, 6, 12, 13)	

Evaluate the difference in peak heights with and without sample preparation. The reduction in peak height between samples prepared using the PyroMark Q96 Vacuum Workstation compared with PyroMark Control Oligo added directly to the PyroMark Q96 Plate Low should not be more than 20%.

If the values are within the set limits, the system is properly installed. If the results are not as stated above, see "Troubleshooting guide" page 44, for possible causes and actions to be taken. If the troubleshooting guide does not explain the problem, 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).

Protocol: Troubleshooting Procedure for the PyroMark Q96 MD Instrument and PyroMark Q96 Vacuum Workstation

If an unexpected result has been obtained it is crucial to determine whether this is related to the PyroMark Q96 MD Instrument, the PyroMark Q96 Vacuum Workstation, or the assay. This protocol describes how to use the PyroMark Control Oligo to verify the function of the PyroMark Q96 MD Instrument, comparing results of samples prepared with or without the PyroMark Q96 Vacuum Workstation.

Important point before starting

- Assay and run files can be set up using either PyroMark Q96 MD Software or PyroMark CpG Software
- For further information on how to create an Assay Setup and a Run Setup, see the *PyroMark Q96 MD Software Online Help*.
- For further information on how to create an Assay Setup and a Run Setup with the PyroMark CpG software see the *PyroMark CpG Software Online Help*.

Things to do before starting

- Follow the instructions in *PyroMark Q96 MD User Manual* to install the PyroMark Q96 MD.
- The dilution buffer provided with the PyroMark Control Oligo needs to be diluted before use. Prepare 1x dilution buffer by mixing 200 µl of 10x dilution buffer with 1800 µl of high-purity water.
- Place the PyroMark Q96 HS Sample Prep Thermoplate on a heating block at 80°C for use in step 30.
- Allow all required reagents and solutions to reach room temperature (15–25°C) before starting.

Procedure

1. **Set up a simplex entry for the PyroMark Control Oligo by using the PyroMark Q96 MD Software alternatively set up an assay in the PyroMark CpG Software using the parameters below.**
2. **Type the following sequence in "Sequence to Analyze".**
   TAYGGTTTGCA
3. **Manually enter the following "Dispensation Order".**
   ACGTTATCGTTGC

For more information on how to create an Assay Setup file, see the *PyroMark Q96 MD Software Online Help* or the *PyroMark CpG Software Online Help*.

Note: For the CpG assay, ignore the warning about sequence direction that is shown when the sequence to analyze and dispensation order is added.

For a thorough control of the instrument, sample preparation and reagents, additional blanks are included in the dispensing order.

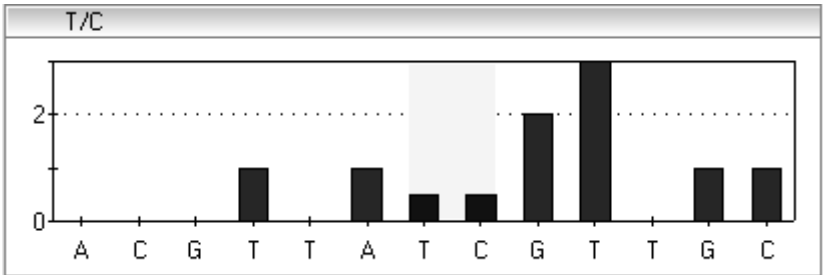

Figure 17. Histogram from PyroMark Q96 MD Software. Nucleotide additions 1, 2, 3, 5 and 11 are blank dispensations and serve as negative controls. The seventh and the eighth dispensations analyze the variable position (wobbled/degenerated base).

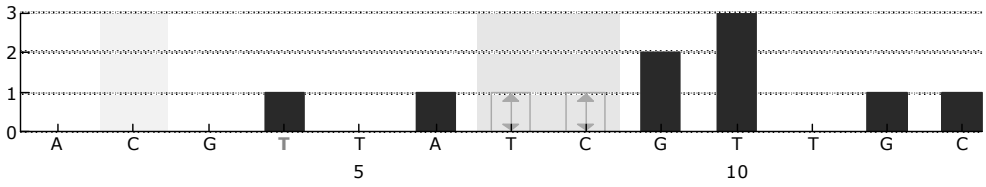

Figure 18. Histogram from PyroMark CpG Software. Nucleotide additions 1, 2, 3, 5 and 11 are blank dispensations and serve as negative controls. The seventh and the eighth dispensations analyze the variable position (wobbled/degenerated base).

4. **Save the entry (PyroMark Q96 MD Software)/assay (PyroMark CpG Software).**
5. **Open a new run and allocate the created entry/assay to appropriate wells. Choose instrument parameters supplied by QIAGEN according to the reagents and cartridge that will be used for the run.**

   We recommend using 16 wells: 8 wells for samples prepared using the PyroMark Q96 Vacuum Workstation and 8 samples added directly to PyroMark Q96 HS Plate.

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

   For more information on how to create a Run Setup file in PyroMark Q96 MD Software, see the *PyroMark Q96 MD Software Online Help*. For more information on how to create a Run Setup file in PyroMark CpG Software, see the *PyroMark CpG Software Online Help*.

6. **Save the Run Setup.**
7. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup.**

   Note: To generate a list of required volumes in the PyroMark Q96 MD Software click **View,** and **Run** in the browser area.

   To generate a list of required volumes in the PyroMark CpG Software, open the run file and select **Volume Information** from the **Tools** menu.
8. **Prepare a master mix for DNA immobilization according to Table 8. Prepare a volume 10% greater than that required for the total number of reactions to be performed.**

   Note: Gently shake the bottle containing Streptavidin Sepharose High Performance until it is a homogeneous solution.

Table 8. Master mix for DNA immobilization

Component	Volume/sample
Streptavidin Sepharose High Performance	2 µl
PyroMark Binding Buffer	40 µl
High-purity water	26 µl
Total volume	**68 µl**

9. **Dilute the PyroMark Control Oligo to 0.06 µM as shown in Table 9.**

Table 9. Dilution of the PyroMark Control Oligo

Component	Volume	Concentration
PyroMark Control Oligo	10 µl	20 µM
1x Dilution buffer*	90 µl	–
First serial dilution	**100** µl	**2 µM**
First serial dilution (from above)	10 µl	2 µM
1x Dilution buffer*	340 µl	–
Final dilution	**350 µl**	**0.06 µM**

* Make sure that the 10x dilution buffer supplied with the PyroMark Control Oligo is diluted with high-purity water before use. See "Things to do before starting", page 36.

10. **Add 68 µl of the master mix and 12 µl of the diluted (0.06 µM) PyroMark Control Oligo to 8 wells of a 96-well PCR plate or strips.**
11. **Seal the PCR plate (or strips) using strip caps.**
12. **Agitate the PCR plate at room temperature (15–25°C) for 5–10 min at 1400 rpm.**

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

    During this step, prepare the PyroMark Q96 Vacuum Workstation for sample preparation (see Appendix B, page 54).
13. **Add 12 µl of PyroMark Annealing Buffer to the wells of the PyroMark Q96 HS Plate that will be used with the immobilized PyroMark Control Oligo to be processed with the PyroMark Q96 Vacuum Workstation. Add 12 µl of the diluted (0.06 µM) PyroMark Control Oligo to 8 additional wells according to the Run Setup.**

    Note: Since the oligonucleotide is self-primed, no sequencing primer is required. The beads are released into PyroMark Annealing Buffer.
14. **Place the PCR plate (or strips) and the PyroMark Q96 HS Plate on the worktable of the PyroMark Q96 Vacuum Workstation (see Figure 19).**

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

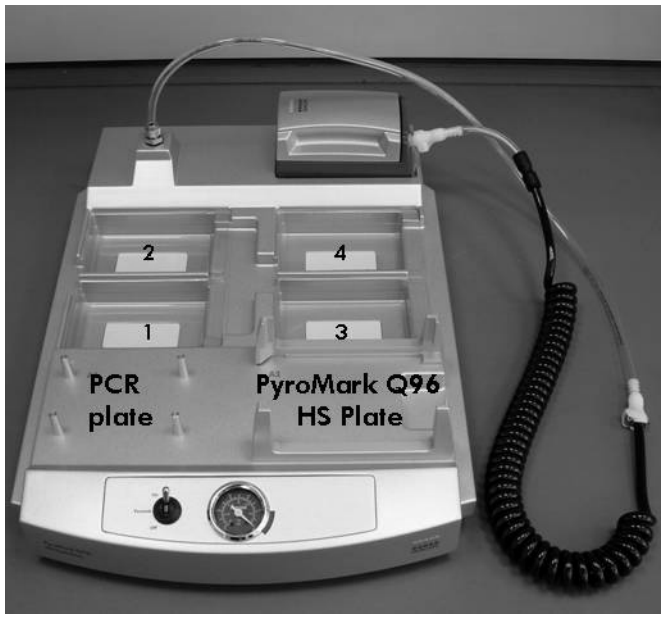

Figure 19. Placement of PCR plate (or strips) and PyroMark Q96 HS Plate on the PyroMark Q96 Vacuum Workstation. The marked positions contain 70% ethanol (1), PyroMark Denaturation Solution (2), PyroMark Wash Buffer (3), and high-purity water (4).

15. **Apply vacuum to the tool by opening the vacuum switch on the vacuum workstation.**

16. **Wash the filter probes by lowering the probes into high-purity water (parking position). Let approximately 180 ml of water flush through the filter probes; i.e. empty the trough.**
17. **Refill the parking position with 180 ml high-purity water.**
18. **Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool.**

    Note: Sepharose beads sediment quickly. If more than 1 min has elapsed since the plate (or strips) was agitated, agitate again for 1 min before capturing the beads.
19. **Transfer the tool to the trough containing 70% ethanol (trough 1). Flush the filter probes for 5 s.**
20. **Transfer the tool to the trough containing PyroMark Denaturation Solution (trough 2). Flush the filter probes for 5 s.**
21. **Transfer the tool to the trough containing PyroMark Wash Buffer (trough 3). Flush the filter probes for 10 s.**
22. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes.**

Figure 20. Illustration of the vacuum tool raised to beyond 90° vertical.

23. **While the tool is held over the PyroMark Q96 HS Plate, close the vacuum switch on the vacuum workstation (Off).**
24. **Release the beads in the plate containing 12 µl PyroMark Annealing Buffer by shaking the tool from side to side. Allow the filter probes to rest on the bottom of the wells.**
25. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**

26. **Wash the filter probes by lowering the probes into the second trough with high-purity water (parking position) and applying vacuum. Flush the probes with 180 ml high-purity water.**
27. **Raise the tool up and back, beyond 90° vertical, for 5 s to drain liquid from the filter probes (see Figure 20).**
28. **Turn off the vacuum switch on the vacuum workstation (Off), and place the tool in the Parking (P) position.**
29. **Turn off the vacuum pump.**

    Note: At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q96 Vacuum Workstation should be checked for dust and spillage, see Appendix C, page 55.
30. **Heat the PyroMark Q96 HS Plate with the samples at 80°C for 2 min using a heating block and the prewarmed PyroMark HS Q96 Sample Prep Thermo Plate Kit.**

    Note: Use one Sample Prep Thermo Plate as lid on the plate to prevent evaporation of the samples.
31. **Remove the PyroMark Q96 HS Plate from the thermo plate, and let the samples cool to room temperature (15–25°C) for at least 5 min.**
32. **Load the nucleotide and reagent tips in the PyroMark Q96 Dispensing Tip Holder with the appropriate volumes of PyroMark Gold Q96 Reagents.**

    Note: To generate a list of required volumes in the PyroMark Q96 MD Software see **View**, and **Run**.
    To generate a list of required volumes in the PyroMark CpG Software, open the run file and select **Volume Information** from the **Tools** menu.
33. **Switch on the instrument.**
34. **Open the process chamber lid using either software.**
35. **Place the PyroMark Q96 HS Plate on the heating block. Close the process chamber lid.**
36. **Open the dispensing unit cover by releasing the latch. With the two RDTs furthest away from you, insert the filled dispensing tip holder into position.**
37. **Close the dispensing unit cover. Make sure that the latch snaps into its locked position.**
38. **Close the instrument lid.**
39. **Perform a run (see *PyroMark Q96 MD User Manual* or *PyroMark CpG Software Online Help*).**
40. **After run open the instrument lid.**
41. **Open the dispensing unit and remove the dispensing tip holder and the PyroMark Q96 HS Plate.**

42. **Close the dispensing unit and the instrument lid (see *PyroMark Q96 MD User Manual* or *PyroMark CpG Software Online Help*).**
43. **Discard the PyroMark Q96 Plate and clean the tips in the PyroMark Q96 Dispensing Tip Holder (see the *PyroMark Gold Q96 Reagents Handbook*).**
44. **Open the run in the PyroMark Q96 MD Software and analyze all wells. The peak pattern should look like the one in Figure 21.**

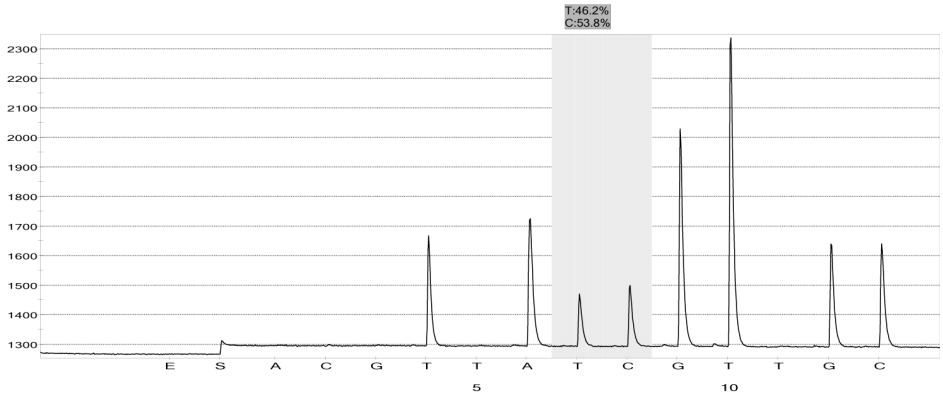

Figure 21. Pyrogram trace from PyroMark MD Software, AQ mode.

45. **Confirm the proper installation of the system and use of the reagents by evaluating the quality assessment, quantification results, background and difference in peak height with and without sample preparation.**

    PyroMark Q96 MD Software: Select all wells by holding down the Ctrl or Shift key and click in the wells to mark them. Selected wells appear with a square around them. Click the arrow next to the **Peak Heights Button** on the **Peak Heights Tab**. Select **Export** from the drop-down list and save the data. Open this file in Microsoft Excel (Delimited) and calculate the mean single peak height and background for each well see below).

    PyroMark CpG Software: Select **Peak Heights** from the **Reports** menu. Save the data in a suitable format (.csv or .tsv). Open this file in Microsoft Excel (Delimited) and calculate the mean single peak height and background for each well (see below).

    Perform a quality assessment.
    All wells should give "Passed" quality. If the quality assessment is "Check" or "Failed", look in "Well info" (PyroMark Q96 MD Software) "Well Information" (PyroMark CpG Software) for explanations.

    Evaluate the quantification results.

    PyroMark Q96 MD Software: Quantification results with standard deviation are shown in **AQ Statistics** under the **Statistics Tab**. Select all wells by holding down the Ctrl or Shift key and click in the wells one-by-one to mark

them. Selected wells appear with a square around them. Click the Statistics button under the Statistics Tab.

PyroMark CpG Software: Quantification results with standard deviation are shown in the **CpG Statistics Report** that can be selected from the **Reports** menu.

The %C should be in the range 40 to 60%. The standard deviation should not exceed 2% units.

Evaluate single peak heights.
The mean single peak height should be at least 350 RLU.

Mean single peak height =	Sum single peaks (dispensation 4, 6, 12, 13)
	4

Evaluate the background.
Background from blank dispensations should not exceed 3%.

Background (%) =	Sum blanks (dispensation 1, 2, 3, 5)	x 100
	Sum single peaks (dispensation 4, 6, 12, 13)	

Evaluate the difference in peak heights with and without sample preparation. The reduction in peak height between samples prepared using the PyroMark Q96 Vacuum Workstation compared with PyroMark Control Oligo added directly to the PyroMark Q96 HS Plate should not be more than 20%.

If the values are within the set limits, the system is properly installed. If the results are not as stated above, see "Troubleshooting guide" page 44, for possible causes and actions to be taken. If the troubleshooting guide does not explain the problem, 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).

Troubleshooting Guide

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

Sections are included for each evaluation performed:

- Quality assessment, below
- Quantification results, page 47
- Single peak heights, page 49
- Background, page 51
- Difference in peak height with and without sample preparation, page 52

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

Refer to the *PyroMark Q96 ID User Manual* for general troubleshooting of the PyroMark Q96 ID.

Refer to the *PyroMark Q96 MD User Manual* for general troubleshooting of the PyroMark Q96 MD.

In the text below, the term Dispensing Unit includes Cartridges, NDTs, and CDTs used to dispense reagents in the various systems.

Quality assessment

	Comments and suggestions
Warning from software about broad peaks	
Concentration of PyroMark Control Oligo too high	Follow the relevant protocol. Make sure to dilute the PyroMark Control Oligo in dilution buffer as described in the protocols.

	Comments and suggestions
High substrate peak	
Contaminated sample leads to unusually high consumption of substrate mixture (noted as a high presequencing signal)	Change buffers. Only use buffers that are supplied by QIAGEN or QIAGEN authorized distributors. Use the zoom in function to check if any peaks have been generated (select a section of Pyrogram with the left mouse button).
Poor or incorrect sequence	
a) PyroMark Control Oligo not correctly prepared	Follow the instruction in the protocols for preparing the PyroMark Control Oligo. Make sure to dilute the PyroMark Control Oligo in the dilution buffer as described in the protocols. Make sure that the 10x dilution buffer provided is first diluted to 1x using high-purity water.
b) Incorrect dispensation order	Check that the correct sequence was typed in the Assay Setup.
c) Buffers or reagents incorrectly diluted or incorrectly stored	Follow the instructions supplied with the reagents. Include an empty well (containing only PyroMark Annealing Buffer) in your run to check if background peaks are coming from the nucleotides.
d) Dispensation error (seen, for example, as split peaks)	Clean or replace the PyroMark Q24 or Q96 Cartridge. If the problem remains, contact QIAGEN Technical Services (for contact information, see back cover or visit www.qiagen.com).
e) Blocked Dispensing Unit	Nucleotides are not dispensed correctly due to a blocked needle in the Dispensing Unit. Clean the Dispensing Unit and check that it is working properly.
f) Damaged Dispensing Unit	Discard the Dispensing Unit according to federal, state, and local environmental regulations for disposal of laboratory waste.
g) Annealing time too long	Carry out annealing for the correct time and at the temperatures described in the protocols.

	Comments and suggestions
Small or missing peaks	
a) Insufficient amount of template for immobilization	Make sure to dilute the PyroMark Control Oligo correctly and use the amounts specified in the protocols.
b) Not enough enzyme or substrate for all wells	Fill the Dispensing Unit according to the instructions in the Pre Run Information report.
c) Wells marked in the Run Setup do not agree with sample placement in the plate	Check that you loaded the PyroMark Plate correctly, according to the Run Setup.
d) One or more of the nucleotide compartments in the Dispensing Unit not correctly filled with reagents or nucleotides	Make sure that sufficient reagents are added to the Dispensing Unit. Follow the instructions for use supplied with the products.
e) Dispensation error (seen, for example, as split peaks)	Clean or replace the Dispensing Unit. If the problem remains, contact QIAGEN Technical Services (for contact information, see back cover or visit www.qiagen.com).
f) Blocked Dispensing Unit	Nucleotides are not dispensed correctly due to a blocked needle in the Dispensing Unit. Clean the Dispensing Unit and check that it is working properly. Enzymes or substrates are not dispensed correctly due to a blocked Dispensing Unit (as indicated by a missing presequencing signal and no peaks in the Pyrogram). Clean the Dispensing Unit and check that it is working properly.
g) Damaged Dispensing Unit	Discard the Dispensing Unit according to federal, state, and local environmental regulations for disposal of laboratory waste.
h) Buffers or reagents incorrectly diluted or incorrectly stored	Follow the instructions supplied with the reagents.

	Comments and suggestions
i) PyroMark instrument started without a plate inserted	Clean the heating block and the light guides/lens array according to instructions in the user manual for the instrument.
j) PyroMark Control Oligo not correctly prepared	Follow the instruction in the protocols for preparing the PyroMark Control Oligo. Make sure to dilute the PyroMark Control Oligo in the dilution buffer as described in the protocols. Make sure that the 10x dilution buffer provided is first diluted to 1x using high-purity water.
k) Contaminated sample leads to unusually high consumption of substrate mixture (noted as a high presequencing signal)	Change buffers. Only use buffers that are supplied by QIAGEN or QIAGEN authorized distributors. Use the zoom in function to check if any peaks have been generated (select a section of Pyrogram with the left mouse button).
Warning regarding signal to noise	
Various	See points a) to k) in "Small or missing peaks", above.

Quantification results

	Comments and suggestions
Poor or incorrect sequence	
a) PyroMark Control Oligo not correctly prepared	Follow the instruction in the protocols for preparing the PyroMark Control Oligo. Make sure to dilute the PyroMark Control Oligo in the dilution buffer as described in the protocols. Make sure that the 10x dilution buffer provided is first diluted to 1x using high-purity water.
b) Incorrect sequence to analyze or dispensation order	Check that the correct sequence was typed in the Assay Setup.

	Comments and suggestions
c) Buffers or reagents incorrectly diluted or incorrectly stored	Follow the instructions supplied with the reagents. Include an empty well (containing only PyroMark Annealing Buffer) in your run to check if background peaks are coming from the nucleotides.
d) Dispensation error (seen, for example, as split peaks)	Clean or replace the Dispensing Unit. If the problem remains, contact QIAGEN Technical Services (for contact information, see back cover or visit www.qiagen.com).
e) Blocked Dispensing Unit	Nucleotides are not dispensed correctly due to a blocked needle in the Dispensing Unit. Clean the Dispensing Unit and check that it is working properly.
f) Damaged Dispensing Unit	Discard the Dispensing Unit according to federal, state, and local environmental regulations for disposal of laboratory waste.
g) Annealing time too long	Carry out annealing for the correct time and at the temperatures described in the protocols.
High background	
a) The storage conditions for one or more reagent did not comply with the instructions given in "Storage", page 4	Check the storage conditions and the expiration date of the reagents and use new reagents, if necessary.
b) Reagents have expired	Check the storage conditions and the expiration date of the reagents and use new reagents, if necessary.

Single peak heights

	Comments and suggestions
Poor or incorrect sequence	
a) PyroMark Control Oligo not correctly prepared	Follow the instruction in the protocols for preparing the PyroMark Control Oligo. Make sure to dilute the PyroMark Control Oligo in the dilution buffer as described in the protocols. Make sure that the 10x dilution buffer provided is first diluted to 1x using high-purity water.
b) Incorrect sequence to analyze or dispensation order	Check that the correct sequence was typed in the Assay Setup.
c) Buffers or reagents incorrectly diluted or incorrectly stored	Follow the instructions supplied with the reagents. Include an empty well (containing only PyroMark Annealing Buffer) in your run to check if background peaks are coming from the nucleotides.
d) Dispensation error (seen, for example, as split peaks)	Clean or replace the Dispensing Unit. If the problem remains, contact QIAGEN Technical Services (for contact information, see back cover or visit www.qiagen.com).
e) Blocked PyroMark Dispensing Unit	Nucleotides are not dispensed correctly due to a blocked needle in the Dispensing Unit. Clean the Dispensing Unit and check that it is working properly.
f) Damaged PyroMark Dispensing Unit	Discard the Dispensing Unit according to federal, state, and local environmental regulations for disposal of laboratory waste.
g) Annealing time too long	Carry out annealing for the correct time and at the temperatures described in the protocols.
Small or missing peaks	
a) Insufficient amount of template for immobilization	Make sure to dilute the PyroMark Control Oligo correctly and use the amounts specified in the protocols.
b) Not enough enzyme or substrate for all wells	Fill the Dispensing Unit according to the instructions in the Pre Run Information report.

	Comments and suggestions
c) Wells marked in the Run Setup do not agree with sample placement in the plate	Check that you loaded the PyroMark Q24 or Q96 Plate correctly, according to the Run Setup.
d) One or more of the nucleotide compartments in the Dispensing Unit not correctly filled with reagents or nucleotides	Make sure that sufficient reagents are added to the Dispensing Unit. Follow the instructions for use supplied with the products.
e) Dispensation error (seen, for example, as split peaks)	Clean or replace the Dispensing Unit. If the problem remains, contact QIAGEN Technical Services (for contact information, see back cover or visit www.qiagen.com).
f) Blocked Dispensing Unit	Nucleotides are not dispensed correctly due to a blocked needle in the Dispensing Unit. Clean the Dispensing Unit and check that it is working properly. Enzymes or substrates are not dispensed correctly due to a blocked Dispensing Unit (as indicated by a missing presequencing signal and no peaks in the Pyrogram). Clean the Dispensing Unit and check that it is working properly.
g) Damaged Dispensing Unit	Discard the Dispensing Unit according to federal, state, and local environmental regulations for disposal of laboratory waste.
h) Buffers or reagents incorrectly diluted or incorrectly stored	Follow the instructions supplied with the reagents.
i) PyroMark Control Oligo not correctly prepared	Follow the instruction in the protocols for preparing the PyroMark Control Oligo. Make sure to dilute the PyroMark Control Oligo in the dilution buffer as described in the protocols. Make sure that the 10x dilution buffer provided is first diluted to 1x using high-purity water.

	Comments and suggestions
j) Contaminated sample leads to unusually high consumption of substrate mixture (noted as a high presequencing signal)	Change buffers. Only use buffers that are supplied by QIAGEN or QIAGEN authorized distributors. Use the zoom in function to check if any peaks have been generated (select a section of Pyrogram with the left mouse button).
Very high peaks	
PyroMark Control Oligo not correctly prepared	Follow the instruction in the protocols for preparing the PyroMark Control Oligo. Make sure to dilute the PyroMark Control Oligo in the dilution buffer as described in the protocols. Make sure that the 10x dilution buffer provided is first diluted to 1x using high-purity water.

Background

	Comments and suggestions
High background	
a) The storage conditions for one or more reagent did not comply with the instructions given in “Storage”, page 4	Check the storage conditions and the expiration date of the reagents and use new reagents, if necessary.
b) Reagents have expired	Check the storage conditions and the expiration date of the reagents and use new reagents, if necessary.

Difference in peak height with and without sample preparation

	Comments and suggestions
Incorrect sample preparation	
a) Liquid left in some wells or tubes when capturing the beads containing immobilized template to the filter probes	Replace corresponding filter probe in the vacuum tool of the PyroMark Q24 or PyroMark Q96 Vacuum Workstation. See the sample preparation guidelines available at 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).
b) Filter probes not working properly	Check the filter probes. Add 80 µl of high-purity water to each well of a PCR plate. Start the vacuum pump and apply vacuum by opening the vacuum switch (On). Lower the vacuum tool into the PCR plate and wait 10 s. Check that all wells of the PCR plate are empty. If not, replace the failed filter probes and repeat the test.
c) White debris (Streptavidin Sepharose High Performance beads) in some wells or tubes when capturing the beads containing immobilized template to the filter probes	Do not leave the PCR plate, used in the immobilization step, for longer than 1 min after mixing is finished. If necessary, mix for an extra minute before capturing the beads.
d) Leakage of the PyroMark Q24 or Q96 Vacuum Workstation	Ensure that the tubing is connected properly and that there is no leakage. The waste filter might be wet and need to be replaced.

Appendix A: Preparation of the PyroMark Q24 Vacuum Workstation

This protocol is a description of how to prepare the PyroMark Q24 Vacuum Workstation before using it for preparation of single-stranded DNA.

Procedure

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

   A suggested setup is shown in Figure 22. Refill the troughs to these levels whenever necessary.

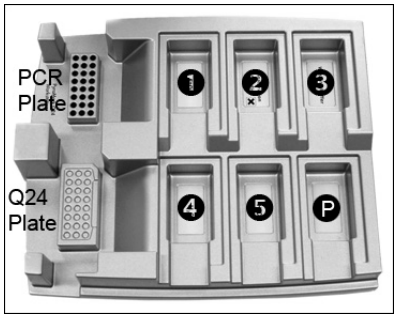

Figure 22. Positions on the PyroMark Q24 Vacuum Workstation.

2. **Switch on the vacuum pump.**
3. **Apply vacuum to the tool by opening the vacuum switch.**
4. **Wash the filter probes by lowering the probes into high-purity water (trough 5). Flush the probes with 70 ml high-purity water. Make sure that the water is being transferred to the waste container. If it is not, then make sure that the tubing is connected correctly and is not broken. Broken tubing should be replaced, see "Replacing the tubing" in the *PyroMark Q24 User Manual*.**
5. **Make sure that the waste filter is dry. If the filter is wet, it should be replaced, see "Replacing the waste filter" in the *PyroMark Q24 User Manual*.**
6. **Refill trough 5 with 70 ml high-purity water.**
7. **Close the vacuum switch on the tool (Off) and place the tool in the Parking (P) position.**

Appendix B: Preparation of the PyroMark Q96 Vacuum Workstation

This protocol is a description of how to prepare the PyroMark Q96 Vacuum Workstation before using it for preparation of single-stranded DNA.

1. **Fill 5 separate troughs (supplied with the PyroMark Q96 Vacuum Workstation) as follows.**

- **Approximately 110 ml ethanol (70%) (1)**
- **Approximately 90 ml PyroMark Denaturation Solution (2)**
- **Approximately 110 ml PyroMark Wash Buffer (3)**
- **Approximately 110 ml high-purity water (4)**
- **Approximately 180 ml high-purity water (Parking position)**

A suggested setup is shown in Figure 23. Refill the troughs to these levels whenever necessary.

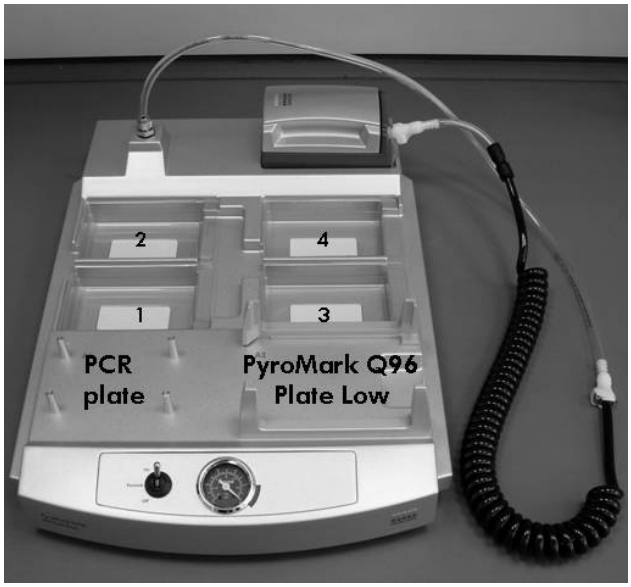

Figure 23. Placement of PCR plate (or strips) and PyroMark Q96 Plate Low or PyroMark Q96 HS Plate on the PyroMark Q96 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4**).

2. **Refill the troughs to approximately these levels whenever needed.**
3. **Start 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 (Parking position). Let approximately 180 ml of water flush through the filter probes, i.e. empty the trough. Ensure that the water is being transferred to the waste container. If not ensure that the tubing is connected properly and there is no leakage.**
6. **Refill Parking Position with 180 ml of high-purity water.**

7. **Switch off the vacuum switch on the vacuum workstation (Off) and place the tool in the Parking (P) position.**

Appendix C: Emptying the Waste Container and Troughs

WARNING 	**Hazardous chemicals** The PyroMark Denaturation Solution used with the PyroMark Q24 Vacuum Workstation contains sodium hydroxide, which is irritating to eyes and skin. Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Material Safety Data Sheets (MSDs) or OSHA,*ACGIH,† or COSHH‡ documents. Venting for fumes and disposal of wastes must be in accordance with all national, state and local health and safety regulations and laws.

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

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

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

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

The following item is required:

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

Procedure

1. **Ensure that no vacuum is applied to the vacuum tool, the vacuum switch is closed (Off), and the vacuum pump is switched off.**
2. **Discard any solutions left in the troughs.**
3. **Rinse the troughs with high-purity water, or replace them, if necessary.**
4. **Empty the waste container.**
   The cap can be removed without disconnecting the tubing.
5. **If the PyroMark Q24 Vacuum Workstation must be cleaned (for dust or spillage), follow the instructions in "Cleaning the PyroMark Q24 Vacuum Workstation" in the *PyroMark Q24 User Manual*.**

References

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

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Ordering Information

Product	Contents	Cat. no.
PyroMark Control Oligo	For installation check of PyroMark systems	979203
Accessories		
PyroMark Gold Q24 Reagents (5 x 24)	For 5 x 24 samples for use on the PyroMark Q24: Enzyme Mixture, Substrate Mixture, and Nucleotides	971802
PyroMark Annealing Buffer (250 ml)*	For annealing sequencing primer to single-stranded PCR product and for Pyrosequencing reaction	979009
PyroMark Binding Buffer (200 ml)*	For binding of biotinylated PCR product to Sepharose beads	979006
PyroMark Denaturation Solution (500 ml)*	For denaturation of double-stranded PCR product into single-stranded template DNA	979007
PyroMark Wash Buffer, concentrate (200 ml)†	For washing of single-stranded DNA	979008
PyroMark Q24 Plate (100)	24-well sequencing reaction plate	979201
PyroMark Q24 Cartridge (3)	Cartridges for dispensing nucleotides and reagents	979302
PyroMark Gold Q96 Reagents (5 x 96)	For performing Pyrosequencing reactions on the PyroMark Q96 ID (5 x 96) and PyroMark Q96 MD (15 x 96)	972804
PyroMark Q96 Plate Low (100)	96-well sequencing reaction plate, for use with PyroMark Q96 ID, 100 plates in each package	979002
PyroMark Q96 HS Plate (100)	96-well sequencing reaction plate, for use with PyroMark Q96 MD, 100 plates in each package	979101
PyroMark Q96 Cartridge	Cartridges for dispensing nucleotides and reagents	979004

* For use with PyroMark Q24, PyroMark Q96 MD, and PyroMark Q96 ID.

† For use with PyroMark Q24 Vacuum Workstation and PyroMark Q96 Vacuum Workstation.

Ordering Information

Product	Contents	Cat. no.
PyroMark Q96 HS Reagent Tip (4)	Reusable tips (4 in each package); for dispensing reagents (RDTs); for use with PyroMark Q96 MD.	979102
PyroMark Q96 HS Nucleotide Tip (8)	Reusable tips (8 in each package); for dispensing nucleotides (NDTs); for use with PyroMark Q96 MD.	979103
Related products		
PyroMark Q24 Instrument	Sequence-based detection platform for Pyrosequencing of 24 samples in parallel	9001514
PyroMark Q24 Software	Application software for PyroMark Q24	9019062
PyroMark Q24 Vacuum Workstation	Workstation for preparing single-stranded DNA from 24 samples	Varies*
PyroMark Q24 Validation Oligo	For performance check of system	979204
PyroMark Q96 ID Instrument	Sequence-based detection platform for Pyrosequencing of 96 samples in parallel	9001525
PyroMark Q96 ID Software	Application software for PyroMark Q96 ID	9019083
PyroMark Q96 MD Instrument	Sequence-based detection platform for Pyrosequencing of 96 samples in parallel	9001526
PyroMark Q96 MD Software	Application software for PyroMark Q96 MD	9019085
PyroMark Q96 Vacuum Workstation (220 V)	For preparation of single-stranded DNA from 96 samples; for use with PyroMark Q96 ID or PyroMarkQ96 MD	9001529
PyroMark Q96 Vacuum Workstation (110 V)	For preparation of single stranded DNA template ready for sequencing by PyroMark Q96 ID or MD. UK	9001528

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

Ordering Information

Product	Contents	Cat. no.
PyroMark PCR Kit (200)*	For 200 reactions: 2x PyroMark PCR Master Mix (includes HotStarTaq DNA Polymerase and optimized PyroMark Reaction Buffer containing 3 mM $MgCl_2$ and dNTPs), 10x CoralLoad® Concentrate, 5x Q-Solution®, 25 mM $MgCl_2$, and RNase-Free Water	978703
EpiTect® Bisulfite Kit (48)*	48 EpiTect Bisulfite Spin Columns, Reaction Mix, DNA Protect Buffer, Carrier RNA, Buffers	59104
EpiTect PCR Control DNA Set (100)*	Human control DNA set (containing both bisulfite-converted methylated and unmethylated DNA and unconverted unmethylated DNA) for 100 control PCRs	59695

* Other kit sizes/formats, available; see www.qiagen.com.

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Limited License Agreement

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