December 2010

PyroMark® Q96 CpG LINE-1 Handbook

For quantification of methylation level of the LINE-1 retrotransposable element

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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Contents

Kit Contents

PyroMark Q96 CpG LINE-1	
Catalog no.	**973043**
Number of reactions	**2 x 96**
Forward and Reverse PCR Primers for amplification of a region in the LINE-1 gene	2 vials
Sequencing Primer for LINE-1	1 vial
Handbook	1

Shipping and Storage

PyroMark Q96 CpG LINE-1 is shipped on dry ice and should be stored at –20°C upon arrival. Dissolved primers should be stored at –20°C.

Product Use Limitations

PyroMark Q96 CpG LINE-1 is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

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

Product Warranty and Satisfaction Guarantee

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

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Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding PyroMark Q96 CpG LINE-1 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 Q96 CpG LINE-1 is tested against predetermined specifications to ensure consistent product quality.

Safety Information

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

CAUTION: Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g., laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Material Safety Data Sheets (MSDSs) or OSHA,* ACGIH,† or COSHH‡ documents. Venting for fumes and disposal of wastes must be in accordance with all national, state, and local health and safety regulations and laws.

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

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

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

24-hour emergency information

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

DNA methylation is vital during development. However, aberrant DNA methylation (both hypermethylation and hypomethylation) has been associated with aging, cancer, and other diseases. One-third of DNA methylation occurs in repetitive elements. Therefore, analysis of these repetitive elements can serve as a surrogate marker for global genomic DNA methylation.

Long interspread nuclear element-1 (LINE-1 or L1) sequences are highly repeated human retrotransposon sequences and constitute about 17% of the human genome. The CpG sites in a LINE-1 promoter are normally heavily methylated to prevent retrotransposition. The genome-wide loss of methylation in core CpG sites of the promoter is regarded as a common epigenetic event in malignancies and may play crucial roles in carcinogenesis.

Principle and procedure

The methylation level of the CpG sites in LINE-1 can be used to estimate the global methylation of a human genome. PyroMark CpG LINE-1 uses real-time, sequence-based Pyrosequencing® technology to quantify methylation level of four CpG sites in positions 331 to 305 of LINE-1 (GenBank accession number X58075).

The procedure is comprised of four simple steps:

- Bisulfite conversion of sample DNA. We recommend the EpiTect® Plus Bisulfite Kits (see Ordering Information, page 28) for complete bisulfite conversion with minimal DNA degradation.
- PCR amplification of the region of interest. We recommend the PyroMark PCR Kit (see Ordering Information, page 28) for this amplification, as the provided reagents are optimized for Pyrosequencing analysis.
- Preparation of single-stranded DNA template.
- Sequence analysis of isolated templates using a Pyrosequencing instrument.

PyroMark Q96 CpG LINE-1 contains forward and reverse PCR primers for amplification of a 146 kb fragment using bisulfite-treated DNA as template. The reverse primer is biotinylated and enables isolation of the correct template DNA for the sequencing reaction. The sequencing primer included is used in the subsequent Pyrosequencing reaction for quantification of the methylation level of four individual CpG sites (Figure 1).

Figure 1. Illustration of the PyroMark Q96 CpG LINE-1 assay. PCR primers are shown as solid arrows and the sequencing primer as a dashed arrow. **FP**: Forward primer; **RPB**: Biotinylated reverse primer; **Seq**: Sequencing primer; **Y**: CpG site.

Description of protocols

This handbook provides all necessary information for Pyrosequencing analysis of methylation level of LINE-1 on the PyroMark Q96 MD.

Before beginning, sample DNA must first be bisulfite converted. This process replaces unmethylated cytosine residues with uracil while methylated cytosines remain unchanged, giving rise to two different sequences that can be distinguished. We recommend EpiTect Plus Bisulfite Kits for complete conversion with minimal degradation of the treated DNA.

The first step is to amplify the target DNA by PCR, as described in the protocol "PCR using the PyroMark PCR Kit" (page 10). The LINE-1 Assay or Entry should be set up while the PCR is running, following the instructions in protocol "Assay and Run Setup Using PyroMark CpG Software or PyroMark Q96 MD Software" (page 13) to set up the LINE-1 Entry. Note that you only need to set up the LINE–1 Entry the first time PyroMark Q96 CpG LINE-1 is used, but a new Run must be set up each time the assay is performed. After amplification, follow the protocols "Immobilization of PCR Products to Streptavidin Sepharose HP Beads" (page 15) and "Preparation of Samples for Pyrosequencing Analysis" (page 17) to generate the sequencing templates. Finally, follow the protocol "Quantification of CpG Methylation of LINE-1" (page 20) to perform the Pyrosequencing run and analyze the data.

Equipment and Reagents to Be Supplied by User

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

- PyroMark Q96 MD (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); alternatively, PyroMark Q96 HS Capillary Tip Holder (cat. no. 9019076)
- PyroMark Q96 HS Reagent Tips (4) (cat. no. 979102)
- PyroMark Q96 HS Nucleotide Tips (8) (cat. no. 979103); alternatively, PyroMark Q96 HS Capillary Tips (8) (cat. no. 979104)
- PyroMark Gold Q96 Reagents (5 x 96) (cat. no. 972804)
- PyroMark Q96 Vacuum Workstation (cat. no. varies depending on region, see Ordering Information, page 43)
- PyroMark Binding Buffer (200 ml) (cat. no. 979006)
- PyroMark Denaturation Solution (500 ml) (cat. no. 979007)
- PyroMark Wash Buffer, concentrate (200 ml) (cat. no. 979008)
- PyroMark Annealing Buffer (250 ml) (cat. no. 979009)
- PyroMark PCR Kit (200) (cat. no. 978703)
- Reagents for bisulfite conversion of DNA. We recommend the EpiTect Plus Bisulfite Kit (see Ordering Information, page 28)
- High-purity water (Milli-Q® 18.2 MΩ x cm or equivalent)
- Ethanol (70%)
- Streptavidin Sepharose High Performance (GE Healthcare, cat. no. 17-5113-01; www.gelifesciences.com)
- Plate mixer for immobilization to beads
- Heating block capable of attaining 80–85°C
- 96-well PCR plate or strips (for PyroMark Q96 MD protocols)
- Strip caps
- Pipets (adjustable)
- Sterile pipet tips

Protocol: PCR Using the PyroMark PCR Kit

This protocol describes the setup and cycling conditions for the amplification of bisulfite-converted DNA using the PyroMark PCR Kit. The PCR products are subsequently used for quantification of CpG methylation of LINE-1 by Pyrosequencing analysis.

Important points before starting

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

Procedure

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

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

2. **Set up the reaction according to Table 1 (page 11).**

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

3. **Gently pipet the reaction solution up and down for thorough mixing and dispense appropriate volumes into PCR tubes.**
4. **Add 10–20 ng bisulfite-converted template DNA to the individual PCR tubes.**

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

5. **Program the thermal cycler according to Table 2 (page 11).**

Table 1. Reaction composition using PyroMark PCR Master Mix

Component	Volume/reaction	Final concentration
Reaction mix		
PyroMark PCR Master Mix, 2x	12.5 µl	Contains HotStarTaq DNA Polymerase,1x PyroMark PCR Buffer,* and dNTPs
CoralLoad Concentrate, 10x	2.5 µl	1x
Forward primer	0.5 µl	0.2 µM
Reverse primer	0.5 µl	0.2 µM
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	10–20 ng bisulfite-converted DNA
Total volume	25 µl	

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

Table 2. Optimized cycling protocol for PyroMark PCR Master Mix

			Additional comments
Initial PCR activation step	15 min	95°C	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling:			
Denaturation	30 s	94°C	
Annealing	30 s	50°C	
Extension	30 s	72°C	
Number of cycles	45		
Final extension	10 min	72°C	

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

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

7. **Use 20 µl of PCR product for subsequent Pyrosequencing analysis.**

   We recommend checking the PCR product prior to Pyrosequencing analysis, e.g. by fast analysis on the QIAxcel® or by agarose gel analysis. See the *PyroMark PCR Kit Handbook* for details.

8. **Proceed to protocol "Assay and Run Setup Using PyroMark CpG Software or PyroMark Q96 MD Software", page 13.**

Protocol: Assay and Run Setup Using PyroMark CpG Software or PyroMark Q96 MD Software

This protocol is for creating an Entry to designate the assay parameters and a Run Setup for quantifying CpG methylation levels in LINE-1 using the PyroMark Q96 MD. This process can be done with either PyroMark CpG Software or PyroMark Q96 MD Software. Follow the protocol steps that correspond to the software you are using: Steps 1–7 for PyroMark CpG Software, or steps 8–15 for PyroMark Q95 MD Software.

Important points before starting

- For further information about how to create an Entry or a Run Setup, consult the *PyroMark Q96 MD Software Online Help* or the *PyroMark CpG Software Online Help*.
- If using PyroMark CpG Software, steps 1–2 are only performed the first time the assay is run.
- If using PyroMark Q96 MD Software, steps 8–9 are only performed the first time the assay is run.

Procedure

1. **Set up a simplex Entry for the PyroMark CpG LINE-1 assay in PyroMark CpG Software using the following parameters (for a histogram, see figure below):**

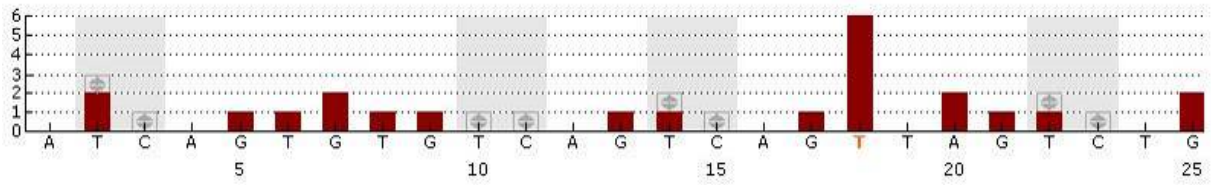
   Sequence to Analyze

   TTYGTGGTGYGTYGTTTTTTAAGTYGGTTT

   Dispensation Order

   ATCAGTGTGTCAGTCAGTTAGTCTG

2. **Save the assay as LINE1.**



Histogram from PyroMark CpG Software.

3. **To create a new run file, click in the toolbar.**

4. **Select instrument parameters according to the instrument, reagent and dispensing tips that will be used for the run.**
5. **Set up the plate.**

   To add an assay to a well, you can either right-click the well and select "Load Assay" from the context menu, or select the assay in the shortcut browser and drag the assay to the appropriate well or selection of wells.
6. **Save the Plate 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 CpG Software, open the run file and select "Volume Information" from the "Tools" menu.
8. **Set up a simplex Entry for the PyroMark CpG LINE-1 assay in PyroMark Q96 MD Software using the following parameters:**

   Sequence to analyze:

   TTYGTGGTGYGTYGTTTTTTAAGTYGGTTT

   Dispensation Order:

   ATCAGTGTGTCAGTCAGTTAGTCTG
9. **Save the entry as LINE1.**
10. **To create a new Run Setup, select "SNP → SNP Runs" in the main menu.**

    The tree view for this submodule opens in the tree view area.
11. **Right-click on a folder in the tree view and select "New SNP Run". The "SNP Run Setup" dialog opens.**

    The Run Setup will be saved in the selected folder.
12. **Define the "Entries" and "Instrument parameters" settings.**
13. **Design the plate layout by entering information about the run and select the instrument parameters and wells to use on the plate.**
14. **Click "Save" to save the Run Setup.**
15. **Print a list of required volumes of enzyme mix, substrate mix, and nucleotides, and the plate setup. Click "View" and choose "Run" in the web browser area.**
16. **Proceed with protocol "Immobilization of PCR Products to Streptavidin Sepharose HP Beads", page 15.**

Protocol: Immobilization of PCR Products to Streptavidin Sepharose HP Beads

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

Things to do before starting

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

Procedure

1. **Gently shake the bottle containing Streptavidin Sepharose HP beads until a homogenous suspension is obtained.**
2. **For each sample, prepare a solution for DNA immobilization as described in Table 3.**

   Note: Prepare a master mix with the components listed in Table 3. Aliquot the master mix to a PCR plate or strips and then add the required volume of PCR product. Adjust the volume of RNase-free water according to the volume of PCR product. Prepare a volume 10% greater than the number of samples to be analyzed.

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

Table 3. DNA immobilization components

Master mix component	Volume per sample
Streptavidin Sepharose HP beads	2 µl
PyroMark Binding Buffer	40 µl
RNase-free water	18 µl
PCR product	**20 µl**
Total volume	**80 µl**

3. **Seal the PCR plate using strip caps.**

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

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

   During immobilization, prepare the PyroMark Q96 Vacuum Workstation for sample preparation (see Appendix A, page 24).

5. **Proceed immediately with the protocol "Preparation of Samples for Pyrosequencing Analysis", page 17.**

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

Protocol: Preparation of Samples for Pyrosequencing Analysis

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

Important point before starting

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

Things to do before starting

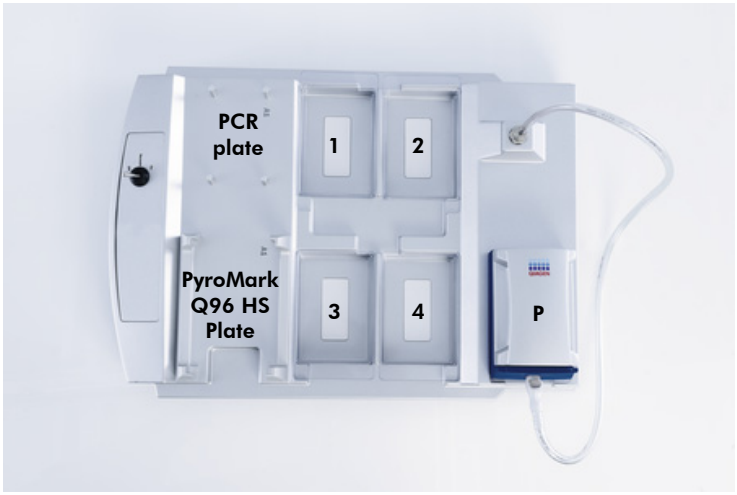
- Before opening the tubes with sequencing primers, spin briefly to collect contents at the bottom of the tube.
- Dissolve the sequencing primer in 180 µl high-purity water (Milli-Q 18.2MΩ x cm or equivalent, filtered through 0.22 µm filter) to a concentration of 10 µM.
- Dilute the sequencing primer to 0.3 µM in Annealing Buffer.
- Prepare the PyroMark Q96 Vacuum Workstation as described in Appendix A, page 24.

Procedure

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

   Ensure that the plate is in the same orientation as when samples were loaded.
3. **Apply vacuum to the tool by opening the vacuum switch.**
4. **Carefully lower the filter probes into the PCR plate (or strips) to capture the beads containing immobilized template. Hold the probes in place for 15 s. Take care when picking up the tool.**

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



PyroMark Q96 Vacuum Workstation. The marked positions contain 70% ethanol (**1**), PyroMark Denaturation Solution (**2**), PyroMark Wash Buffer (**3**), and high-purity water (**4** and **P**).

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

Vacuum tool raised beyond 90° vertical.

9. **While holding the tool over the PyroMark Q96 HS Plate, close the vacuum switch on the tool (Off).**
10. **Release the beads into the wells containing sequencing primer by gently shaking the tool in the wells.**

11. **Transfer the tool to the trough containing high-purity water (trough 4) and agitate the tool for 10 s.**
12. **Wash the filter probes by lowering the probes into high-purity water (parking position, P) and applying vacuum. Flush the probes with 70 ml high-purity water.**
13. **Raise the tool beyond 90° vertical, for 5 s to drain liquid from the filter probes.**
14. **Close the vacuum switch on the tool (Off), and place the tool in the Parking (P) position.**
15. **Turn off the vacuum pump.**

    Note: At the end of a working day, liquid waste and remaining solutions should be discarded and the PyroMark Q96 Vacuum Workstation should be checked for dust and spillage, see Appendix B, page 26.
16. **Heat the PyroMark Q96 HS Plate with the samples at 80°C for 2 min using a heating block and the prewarmed PyroMark HS Q96 Sample Prep Thermo Plate.**

    Note: Use one Sample Prep Thermo Plate as a lid on the plate to prevent sample evaporation.
17. **Remove the PyroMark Q96 HS Plate from the thermo plate, and allow the samples to cool to room temperature (15–25°C) for at least 5 min.**
18. **Proceed with the protocol "Quantification of CpG Methylation in LINE-1", page 20.**

Protocol: Quantification of CpG Methylation in LINE–1

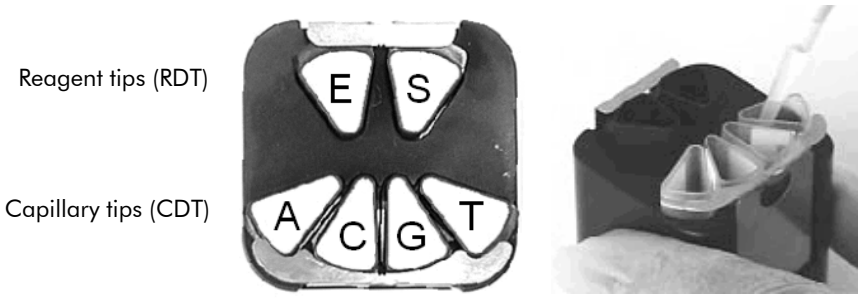
This protocol describes loading of PyroMark Gold Q96 Reagents into the PyroMark Q96 HS Reagent Tips (RDTs) and Capillary Tips (CDTs) and analysis of LINE-1 methylation using the PyroMark Q96 MD. If using the PyroMark Q96 HS Nucleotide Tips and PyroMark Q96 Dispensing Tip Holder, please refer to the *PyroMark Q96 HS Nucleotide Tip Product Sheet* for filling instructions. For a detailed description about how to set up a run, see *PyroMark Q96 MD Online Help*.

Things to do before starting

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

Protocol

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

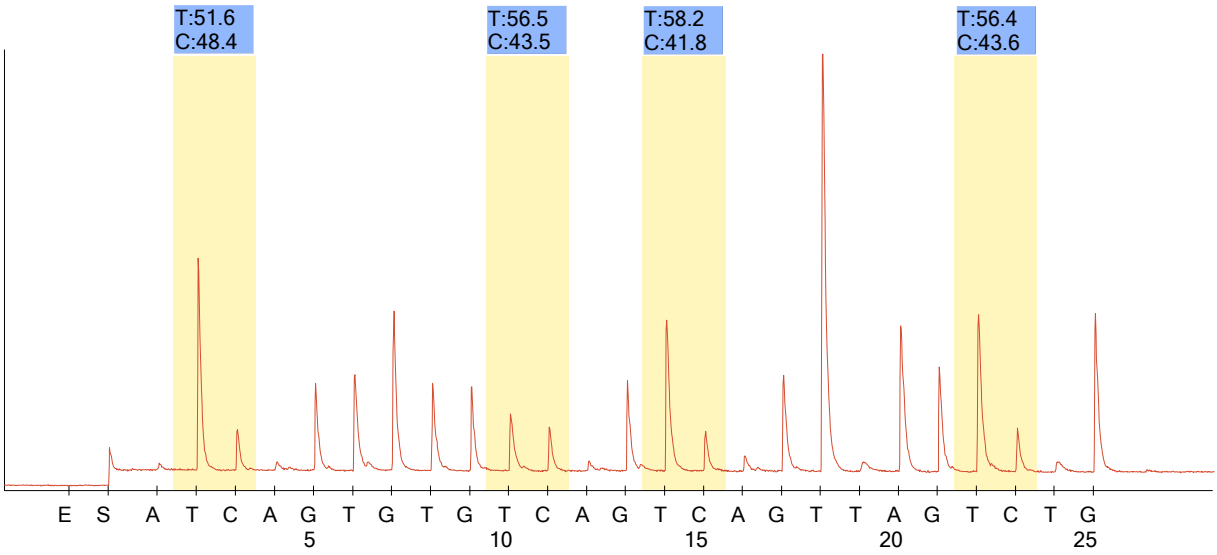


Arrangement of tips in the PyroMark Q96 Capillary Tip Holder.
E: Enzyme Mixture; **S**: Substrate Mixture; **G**: dGTP; **C**: dCTP; **T**: dTTP; **A**: dATPαS.

2. **Open the processing chamber lid using the software.**
3. **Place the PyroMark Q96 HS Plate on the heating block. Close the process chamber lid.**
4. **Open the dispensing unit cover by releasing the latch. With the two RDTs furthest away from you, insert the filled dispensing tip holder into position.**

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

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



Pyrogram trace obtained after analysis of samples. The analyzed CpG sites are highlighted with a gray background.

Troubleshooting Guide

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

	Comments and suggestions
Low or missing peaks in the Pyrogram	
a) PCR failed	Check the PCR samples using a gel technique to confirm that there is one strong, specific band. If not, rerun the PCR using high-quality DNA.
b) The wells marked in the run setup do not agree with the sample placement on the plate for immobilization	Check that the PCR plate (or strips) was loaded on the vacuum workstation according to the plate setup.
c) One or several of the dispensing tips were not correctly filled	Be sure to add sufficient reagents in the correct dispensing tip.
d) One of the tip needles is blocked or damaged	Check that the dispensing tips are working correctly. For detailed instructions, see Section 7.9 of the *PyroMark Q96 MD User Manual*. In case of bent needles, discard the dispensing tip according to federal, state, and local environmental regulations for disposal of laboratory waste.
e) The tip holder is inserted incorrectly	Ensure that the tip holder is inserted correctly.
f) Low signal due to dirty lens array	Clean the heating block and lens array; see sections 7.4 and 7.5 of the *PyroMark Q96 MD User Manual*.

	Comments and suggestions
g) Filter probes not working correctly	Test the filter probes and ensure they are working correctly. See section 7.12 of the *PyroMark Q96 MD User Manual*.
Poor or faulty sequence	
a) Incorrect sequence to analyze	Check typing and reference sequence.
b) Nucleotides incorrectly diluted or stored	Be sure to follow the instructions in the *PyroMark Gold Q96 Reagents Handbook*.
c) Crosstalk (light from one well appears in the neighboring well)	Avoid placing assays with high signals close to assays with low signals.
d) Dispensation error	Replace the dispensing tips. If the problem remains, contact QIAGEN Technical Service.
Software warning: Uncertain/failed bisulfite conversion at dispensation 2	
a) Failed or incomplete bisulfite treatment	Ensure that sample DNA is fully converted during incubation with sodium bisulfite. We recommend the EpiTect Plus Bisulfite Kits (see Ordering Information, page 28) for complete bisulfite conversion with minimal DNA degradation.

Appendix A: Preparation of the PyroMark Q96 Vacuum Workstation

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

Important point before starting

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

Procedure

1. **Fill five separate troughs (supplied with the PyroMark Q96 Vacuum Workstation) according to Table 4.**

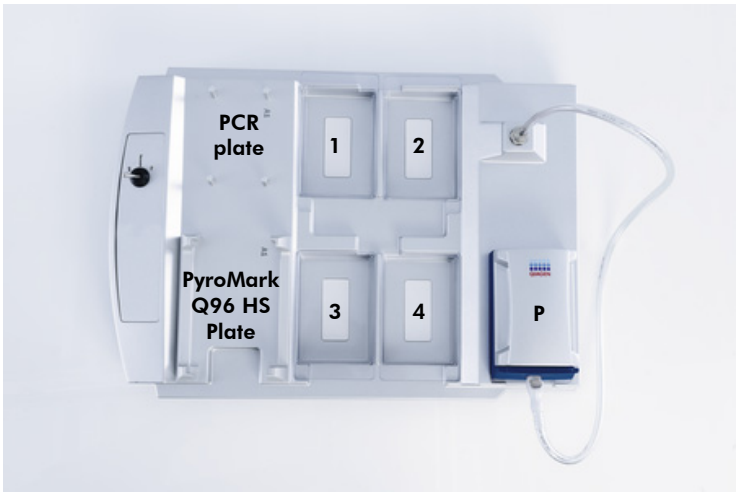
   A suggested workstation setup is shown in the figure on the next page. Refill the troughs to these levels whenever necessary.

Table 4. Vacuum workstation volumes

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

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 the Parking Position (trough P) and flushing them with 180 ml high-purity water.**

   Ensure that the water is being transferred to the waste container. If not, ensure that the tubing is connected correctly and is not broken. Broken tubing should be replaced, see the *PyroMark Q96 User Manual* section on replacing the tubing.



PyroMark Q96 Vacuum Workstation.

5. **Refill trough 5 with 70 ml high-purity water or Parking Position with 180 ml high-purity water.**
6. **Close the vacuum switch (Off) and place the tool in the Parking (P) position.**

Appendix B: Emptying the Waste Container and Troughs

WARNING	Hazardous chemicals
	The Denaturation Solution used with the PyroMark Q24 Vacuum Workstation or PyroMark Q96 Vacuum Workstation contains sodium hydroxide, which is irritating to eyes and skin. Always wear safety glasses, gloves, and a lab coat. The responsible body (e.g. laboratory manager) must take the necessary precautions to ensure that the surrounding workplace is safe and that the instrument operators are not exposed to hazardous levels of toxic substances (chemical or biological) as defined in the applicable Material Safety Data Sheets (MSDSs) or OSHA,*ACGIH,† or COSHH‡ documents. Venting for fumes and disposal of wastes must be in accordance with all national, state and local health and safety regulations and laws.

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

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

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

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

The following item is required:

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

Procedure

1. **Ensure that no vacuum is applied to the vacuum tool, the vacuum switch is closed (Off), and the vacuum pump is switched off.**
2. **Discard any solutions left in the troughs.**
3. **Rinse the troughs with high-purity water, or replace them, if necessary.**
4. **Empty the waste container.**
   The cap can be removed without disconnecting the tubing.
5. **If the PyroMark Q96 Vacuum Workstation must be cleaned (for dust or spillage), follow the instructions in relevant manual (*PyroMark Q96 MD 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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Product	Contents	Cat. no.
PyroMark Q96 CpG LINE–1	Methylation detection assay: PCR primers and sequencing primer for quantification of global methylation level in transposable elements	973043
Accessories		
PyroMark Gold Q96 reagents	For performing Pyrosequencing reactions on the PyroMark Q96 MD (15 x 96)	972804
PyroMark Binding Buffer (200 ml)	Solution providing optimal conditions for immobilization of biotinylated DNA to streptavidin-coated Sepharose beads	979006
PyroMark Denaturation Sol. (500 ml)	Solution for use with the PyroMark Q96 Vacuum Workstation for preparation of single stranded DNA template	979007
PyroMark Wash Buffer (conc., 200 ml)	Solution for use with the PyroMark Q24 Vacuum Workstation and PyroMark Q96 Vacuum Workstation to wash and neutralize the immobilized DNA	979008
PyroMark Annealing Buffer (250 ml)	Solution providing optimal conditions for annealing of sequencing primer to DNA template	979009
PyroMark Q96 HS Plate (100)	For sample analysis of DNA template on PyroMark Q96 MD	979101
PyroMark Q96 HS Nucleotide Tips (8)	Reusable tips (8 in each package); for dispensing nucleotides (NDTs); for use with PyroMark Q96 MD	979103
PyroMark Q96 HS Capillary Tips (8)	Reusable capillary tips (8 in each package); for dispensing nucleotides (CDTs); for use with PyroMark Q96 MD	979104
PyroMark Q96 HS Reagent Tips (4)	Reusable tips (4 in each package); for dispensing reagents (RDTs); for use with PyroMark Q96 MD	979102
PyroMark Q96 HS Dispensing Tip Holder	Reusable holder for tips, nucleotide dispensing tips, and reagent dispensing tips, for use with PyroMark Q96 MD	9019075

Product	Contents	Cat. no.
PyroMark Q96 HS Capillary Tip Holder	Reusable holder for tips, nucleotide dispensing tips, and reagent dispensing tips, for use with PyroMark Q96 MD	9019076
EpiTect Plus DNA Bisulfite Kit (48)	48 MinElute DNA spin columns, Bisulfite mix, DNA Protect Buffer, Carrier RNA, Buffers	59124
EpiTect Plus FFPE Bisulfite Kit (48)	48 MinElute DNA spin columns, Bisulfite mix, DNA Protect Buffer, Carrier RNA, Buffers, Deparaffinization Solution, Lysis Buffer FTB	59144
EpiTect Plus LyseAll Bisulfite Kit (48)	48 MinElute DNA spin columns, Bisulfite mix, DNA Protect Buffer, Carrier RNA, Buffers, Lysis Buffer FTB	59164
Related products		
PyroMark Q96 MD	Instrument for analysis of SNPs, mutations, and CpG methylation using Pyrosequencing technology	9019526
PyroMark Q96 MD Software	Application software, for laboratory use only	9019085
PyroMark Q96 Vacuum Workstation	For preparation of single stranded DNA template ready for sequencing by PyroMark Q96	Varies†
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
PyroMark PCR Kit (800)	For 800 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	978705

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

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