July 2024

PyroMark® Q24 CpG MLH1 Handbook

For quantification of methylation level of the MLH1 gene using PyroMark Q24, PyroMark Q24 Advanced, and PyroMark Q48 Autoprep

Sample to Insight

Contents

Kit Contents

PyroMark Q24 CpG MLH1 Kit	
Catalog no.	**970022**
Number of reactions	**96**
PyroMark Forw Primer MLH1	2 vials
PyroMark Rev Primer MLH1	2 vials
PyroMark Seq Primer MLH1	2 vials

Shipping and Storage

The PyroMark Q24 CpG MLH1kit is shipped on dry ice. It should be stored at –20 °C immediately upon arrival. Dissolved Primers should be stored at –20 °C.

Intended Use

The PyroMark Q24 CpG MLH1 kit 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.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS 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 waste 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).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of PyroMark Q24 CpG MLH1 kits are tested against predetermined specifications to ensure consistent product quality.

Introduction

Principle and procedure

The PyroMark Q24 CpG MLH1 assay is used to quantify the methylation of CpG sites of the MLH1 gene by real-time, sequence-based Pyrosequencing®. In this assay the level of methylation is detected for CpG sites in the genomic region chr3:36,993,279-36,993,309 (Reference genome: GRCh38.p14) of the MLH1 gene (Ensembl ID: ENSG00000076242). Determination of methylation requires bisulfite conversion of DNA to convert unmethylated cytosines to uraciles which are replaced by thymines during PCR, and which can be analyzed by Pyrosequencing®. Since methylated cytosines are protected from this conversion, the ratio of cytosines and thymines can be used to determine the methylation level of each CpG site within the target, independently.

The procedure is comprised of four simple steps:

- Bisulfite conversion of sample DNA. We recommend the EpiTect® Fast Bisulfite Kits (cat. no. 59802) for complete bisulfite conversion with minimal DNA degradation.
- PCR amplification of the region of interest. We recommend the PyroMark PCR Kit (cat. no. 978703) 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 Q24 CpG MLH1 contains forward and reverse PCR primers for amplification of a 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 the CpG sites, plus a control for complete conversion of DNA through the bisulfite treatment (Figure 1, next page).

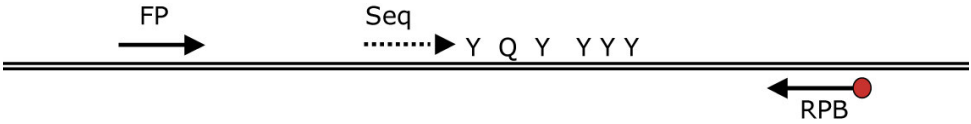

Figure 1. Illustration of the MLH1 assay. PCR primers are shown as solid arrows and the sequencing primer as a dashed arrow. FP, forward primer; RPB, biotinylated reverse primer; Seq, sequencing primer; Y, CpG site; Q, Control for completion of bisulfite treatment.

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 safety data sheets (SDSs), available from the product supplier.

- DNA preparation reagents
- EpiTect Fast Bisulfite Conversion Kit (cat. no. 59802)
- PyroMark PCR Kit (200) (cat. no. 978703)
- High purity water (Milli-Q® 18.2 MΩ × cm or equivalent, filtered through 0.22 µm filter)
- Please refer for further equipment and reagents to be supplied to the respective handbook of the instrument you are using:
  - When using PyroMark Q24, see "5.3 Sample Preparation" section in *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240).
  - When using PyroMark Q24 Advanced, see "5.3 Sample and Reagent Preparation" section in *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341).
  - When using PyroMark Q48 Autoprep, see "Preparing Templates and Reagents" section in *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971).

Materials provided

- Two vials each of Forward MLH1 PCR Primer and Reverse MLH1 PCR Primer.
- Each PCR primer should be dissolved in 120 µL high purity water (Milli-Q 18.2 MΩ × cm or equivalent, filtered through 0.22 µm filter) to give a concentration of 10 µM.
- Two vials of MLH1 Sequencing Primer. Should be dissolved in 180 µL buffer to give a concentration of 10 µM.

  Note:
  - PyroMark Q24: The sequencing primer should be dissolved in Annealing buffer.
  - PyroMark Q24 Advanced: The sequencing primer should be dissolved in Advanced Annealing buffer.

- PyroMark Q48 Autoprep: The sequencing primer should be dissolved in Advanced Annealing buffer. Dilute the sequencing primer further down to a final concentration of 4 µM.

Important Points Before Starting

- For bisulfite conversion, use the EpiTect Fast Bisulfite Conversion Kit and follow the instructions in the handbook.
- Dissolve PCR primers and sequencing primers as described in "Materials provided", page 9. Before opening, spin briefly to collect contents at the bottom of the tube.
- Ensure that the reactions are thoroughly mixed, as well as prepared and incubated at the recommended temperatures.

Description of protocols

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 Fast Bisulfite Kit (cat. no. 59802) for complete conversion with minimal degradation of the treated DNA.

The first step is to amplify the bisulfite converted target DNA by PCR, as described in "Protocol: PCR Using the PyroMark PCR Kit", page 13. The MLH1 Assay should be set up while the PCR is running, following the instructions in protocol "Protocol: Assay and Run Setup", page 16. After amplification, follow the protocols according to the respective instrument's handbook to prepare the sequencing templates for Pyrosequencing analysis:

- *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240): see "5.3 Sample preparation", continuing from "5.3.3 Immobilizing the PCR products to beads", and "5.4 Preparation of PyroMark Gold Q24 Reagents".
- *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341): see "5.3 Sample and reagent preparation", continuing from "5.3.3 Immobilizing the PCR products to beads".

- *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971): see "Preparing templates and reagents", continuing from "6.1.5 Absorber strip".

Finally, follow the protocols according to the respective instrument's handbook to perform the Pyrosequencing run and analyze the data:

- *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240): see "5.5 Processing a run on the PyroMark Q24 Instrument."
- *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341): see "5.4 Processing a run on the PyroMark Q24 Advanced."
- *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971): see "Starting a run" and "7 PyroMark Q48 Autoprep Software".

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 (cat. no. 978703). The PCR products are subsequently used for quantification of CpG methylation of MLH1 by Pyrosequencing analysis.

Important points before starting

- See the *PyroMark PCR Kit Handbook* for more detailed information.
- HotStarTaq® DNA Polymerase requires an activation step of 15 min at 95°C (step 5 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.

Procedure

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

   Important: Mix the solutions before use to avoid localized concentrations of salt.
2. Set up the reaction according to Table 1a and Table 1b, next page. It is not necessary to keep reaction vessels on ice since HotStarTaq DNA Polymerase is inactive at room temperature.
3. Gently pipette the reaction solution up and down for thorough mixing and dispense appropriate volumes into PCR tubes.
4. Add 10 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.

Table 1a. Reaction composition using PyroMark PCR Master Mix for PyroMark Q24 and PyroMark Q24 Advanced

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

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

Table 1b. Reaction composition using PyroMark PCR Master Mix for PyroMark Q48 Autoprep

Component	Volume (µL) per reaction	Final concentration
Reaction Mix		
PyroMark PCR Master Mix, 2x	12.5	Contains HotStarTaq DNA Polymerase,1x PyroMark PCR Buffer*, and dNTPs
CoralLoad Concentrate, 10x	2.5	1x
Forward Primer	1	0.4 µM
Reverse Primer	1	0.4 µM
RNase-free water	Variable	–
Template DNA		
Template DNA, added at step 4	Variable	10 ng bisulfite converted NDA
Total volume	25	–

5. Program the thermal cycler according to Table 2, next page.

Table 2. Optimized cycling protocol for PyroMark PCR Master Mix

	Time	Temperature (°C)	Notes
Initial PCR activation step	15 min	95	HotStarTaq DNA Polymerase is activated by this heating step
3-step cycling			
Denaturation	30 s	94	
Annealing	30 s	56	
Extension	30 s	72	
Number of cycles			45 cycles
Final extension	10 min	72	
Hold	∞	4	

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 for longer storage.

7. Use 10 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. The amplicon length is 181 bp.

8. Proceed to "Protocol: Assay and Run Setup", next page.

Protocol: Assay and Run Setup

This protocol is for setting up the assay parameters and creating a Run Setup for CpG methylation analysis in MLH1.

Please refer to the section below according to the PyroMark instrument used.

PyroMark Q24

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

Procedure

1. **Sequence to Analyze** – Set up the MLH1 Assay by selecting **New CpG Assay** in the PyroMark Q24 Software and enter the following sequence in **Sequence to Analyze**:

   YGGATAGYGATTTTTAAYGYGTAAGYGTATA

2. **Nucleotide Dispensation Order** – Click **Generate Dispensation Order**. Choose the T at dispensation 6 as a control for bisulfite treatment by left-clicking the orange **T**. The following dispensation order should be used:

   GTCGACTATGTCGATTGATCAGTCGTATGTCGTA

   The control for completion of bisulfite treatment is highlighted in grey in the sequence above. It is automatically analyzed by PyroMark Q24 Software and indicated in orange, (see Figure 2, next page).

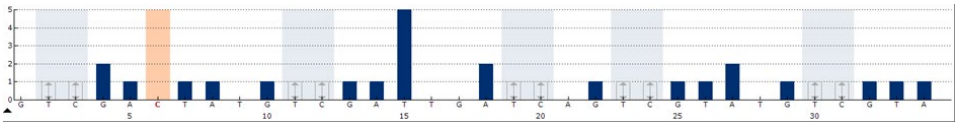

Figure 2. Histogram for the MLH1 assay.

The nucleotide addition of T at dispensation 28 might give a background signal. It is therefore recommended to deselect this dispensation as a reference peak when creating the Assay Setup. Right click in the **Histogram**, tick **Show Reference Peaks**, click the blue diamond above dispensation 28, and save the assay.

3. **Run Setup** – Create a new run file by selecting New Run.
   Set up the plate by adding the MLH1 assay to each used well. Proceed with the Run Setup, preparation of samples, and run according to instructions in PyroMark Q24 User Manual.

PyroMark Q24 Advanced

When using PyroMark Q24 Advanced, please refer to the section below.

Procedure

1. **Sequence to Analyze** – Set up the MLH1 Assay by selecting **New CpG Assay** in the PyroMark Q24 Advanced Software and enter the following sequence in **Sequence Before Bisulfite Treatment (Forward Orientation)**:

   CGGACAGCGATCTCTAACGCGCAAGCGCATA

   The software automatically generates the following Sequence to Analyze:

   YGGATAGYGATTTTTAAYGYGTAAGYGTATA

2. **Nucleotide Dispensation Order** – Click **Generate Dispensation Order**. Bisulfite treatment control will be included automatically. The following dispensation order should have been generated:

GTCGACTATGTCGATTGATCAGTCGTATGTCGTA

The control for completion of bisulfite treatment is highlighted in grey in the sequence above. It is automatically analyzed by PyroMark Q24 Advanced Software and indicated in orange, Figure 3.

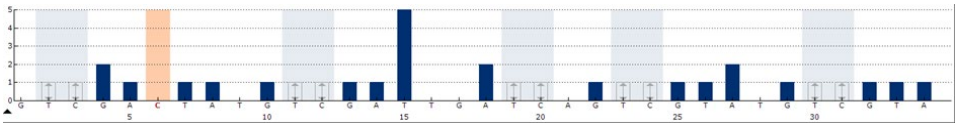

Figure 3. Histogram for the MLH1 assay.

The nucleotide addition of T at dispensation 28 might give a background signal. It is therefore recommended to deselect this dispensation as a reference peak when creating the Assay Setup. Right click in the **Histogram**, tick **Show Reference Peaks**, click the blue diamond above dispensation 28, and save the assay.

3. **Run Setup** – Create a new run file by selecting **New Run**. Set up the plate by adding the MLH1 assay to each used well. Proceed with the Run Setup, preparation of samples, and run according to instructions in *PyroMark Q24 Advanced User Manual*.

PyroMark Q48 Autoprep

When using PyroMark Q48 Autoprep, please refer to the section below.

Procedure

1. **Sequence to Analyze** – Set up the MLH1 Assay by selecting **New CpG Assay** in the PyroMark Q48 Autoprep Software and enter the following sequence in Sequence Before Bisulfite Treatment (Forward Orientation):

CGGACAGCGATCTCTAACGCGCAAGCGCATA

The software automatically generates the following Sequence to Analyze:

YGGATAGYGATTTTTAAYGYGTAAGYGTATA

2. **Nucleotide Dispensation Order** – Click **Generate Dispensation Order**. Bisulfite treatment control will be included automatically. The following dispensation order should have been generated:

   GTCGACTATGTCGATTGATCAGTCGTATGTCGTA

   The control for completion of bisulfite treatment is highlighted in grey in the sequence above. It is automatically analyzed by PyroMark Q48 Autoprep Software and indicated in orange, Figure 4.

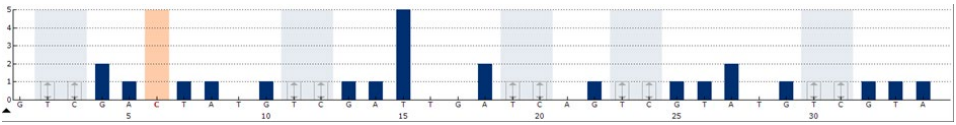

Figure 4. Histogram for the MLH1 assay.

   The nucleotide addition of T at dispensation 28 might give a background signal. It is therefore recommended to deselect this dispensation as a reference peak when creating the Assay Setup. Right click in the **Histogram**, tick **Show Reference Peaks**, click the blue diamond above dispensation 28, and save the assay.

3. **Run Setup** – Create a new run file by selecting **New Run**.
   Set up the plate by adding the MLH1 assay to each used well. Proceed with the Run Setup, preparation of samples and run according to instructions in *PyroMark Q48 Autoprep User Manual*.

Protocol: Sample Preparation

Important point before starting

After amplification, follow the protocols according to the respective instrument's handbook to prepare the sequencing templates for Pyrosequencing analysis:

- When using PyroMark Q24, see the following in *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240):
  - "5.3 Sample preparation", continuing from "5.3.3 Immobilizing the PCR products to beads."
  - "5.4 Preparation of PyroMark Gold Q24 Reagents"

- When using PyroMark Q24 Advanced, see the following in *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341):
  - "5.3 Sample and reagent preparation", continuing from "5.3.3 Immobilizing the PCR products to beads".

- When using PyroMark Q48 Autoprep, see the following in *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971):
  - "Preparing templates and reagents", continuing from "6.1.5 Absorber strip."

Protocol: Performing and Analyzing a Run

Important point before starting

- The sequencing primer stock solution has a concentration of 10 µM.
- Before use on the PyroMark Q48 Autoprep Instrument, an appropriate volume of the stock solution has to be diluted down to a final concentration of 4 µM. For dilution, use the PyroMark Advanced Annealing Buffer.
- Follow the protocols according to the respective instrument's handbook to perform the Pyrosequencing run and analyze the data:

    - When using PyroMark Q24, see the following in *PyroMark Q24 User Manual* (www.qiagen.com/HB-0240):
      "5.5 Processing a run on the PyroMark Q24 Instrument"

    - When using PyroMark Q24 Advanced, see the following in *PyroMark Q24 Advanced User Manual* (www.qiagen.com/HB-1341):
      "5.4 Processing a run on the PyroMark Q24 Advanced"

    - When using PyroMark Q48 Autoprep, see the following in *PyroMark Q48 Autoprep User Manual* (www.qiagen.com/HB-1971):
      "Starting a run"
      "7 PyroMark Q48 Autoprep Software"

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 (for contact information, visit **www.qiagen.com**)

- When using PyroMark Q24, see "Troubleshooting Guide" section 7 in *PyroMark Q24 User Manual*.
- When using PyroMark Q24 Advanced, see "Troubleshooting Guide" section 7 in *PyroMark Q24 Advanced User Manual*.
- When using PyroMark Q48 Autoprep, see "Troubleshooting" section 9 in *PyroMark Q48 Autoprep User Manual*.

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Ordering Information

Product	Contents	Cat. no.
Assays & Controls		
PyroMark Q24 CpG MLH1	For 96 reactions: Forward primer, Reverse primer, and Sequencing primer for the analysis of MLH1 methylation using the PyroMark Q24, PyroMark Q24 Advanced or PyroMark Q48 Autoprep	970022
PyroMark Q24 CpG MGMT	For 96 reactions: Forward primer, reverse primer, and sequencing primer for the analysis of MGMT methylation using the PyroMark Q24, PyroMark Q24 Advanced or PyroMark Q48 Autoprep	970032
PyroMark Control Oligo	For installation check of the system	979203
PCR & Bisulfite Conversion		
PyroMark PCR Kit (200)	PCR Master Mix for PCR reactions optimized for Pyrosequencing analysis	978703
EpiTect Fast DNA Bisulfite Kit (50)	For 50 preps: Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59824
EpiTect Fast FFPE Bisulfite Kit (50)	For 50 preps: Deparaffinization Solution, Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA and Buffers	59844
EpiTect Fast LyseAll Bisulfite Kit (50)	For 50 preps: Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer,	59864

Product	Contents	Cat. no.
	MinElute DNA Spin Columns, Carrier RNA, and Buffers	
PyroMark Q24		
PyroMark Q24	Instrument, for laboratory use only	9001514
PyroMark Q24 Software	Analysis software, for laboratory use only	9019062
PyroMark Q24 Advanced		
PyroMark Q24 Advanced CpG Reagents (4 x 24)	For 4 x 24 samples for use on the PyroMark Q24 Advanced: Enzyme Mixture, Substrate Mixture, Buffers, and Nucleotides for CpG and long-read sequencing runs	970922
PyroMark Q24 Advanced	Instrument, software, and installation for advanced Pyrosequencing analysis of 24 samples in parallel	9002270
PyroMark Q48 Autoprep		
PyroMark Q48 Autoprep	Instrument, software, and pipette	9002470
PyroMark Q48 Discs (50)	50 discs for running PyroMark Q48 Autoprep reactions	974901
PyroMark Q48 Absorber Strips (100)	100 absorber strips for running PyroMark Q48 Autoprep reactions	974912
PyroMark Q48 Autoprep Starter Kit	PyroMark Q48 Magnetic Beads (300), PyroMark Q48 Advanced CpG Reagents (4 x 48), PyroMark Control Oligo, PyroMark Q48 Discs (50), and PyroMark Q48 Absorber Strips (100)	974230
PyroMark Q48 Advanced Reagents (4 x 48)	Reagents for 4 x 48 PyroMark Q48 Autoprep standard reactions	974002

Product	Contents	Cat. no.
PyroMark Q48 Advanced CpG Reagents (4 x 48)	Reagents for 4 × 48 PyroMark Q48 Autoprep CpG and long-read reactions	974022
PyroMark Q48 Magnetic Beads (300)	Magnetic streptavidin-coated Sepharose beads for running 300 PyroMark Q48 Autoprep reactions	974203

Document Revision History

Revision	Description
07/2024	Initial release

Limited License Agreement for PyroMark Q24 CpG MLH1 Kit

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