Quick-Start Protocol

July 2024

PyroMark® Q24 CpG MLH1 Assay

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.

For optimal performance and stability, the PyroMark Q24 CpG MLH1 kit should be shipped on dry ice and stored at –20 °C immediately upon arrival. Dissolved Primers should be stored at –20 °C.

Further information

- *PyroMark® Q24 CpG MLH1 Handbook:* **www.qiagen.com/HB-3540**
- Safety Data Sheets: www.qiagen.com/safety
- Technical assistance: **support.qiagen.com**

Notes before starting

- For bisulfite conversion, use the EpiTect Fast Bisulfite Conversion Kit (cat. no. 59802) and follow the instructions in the handbook.
- For amplification of bisulfite-converted DNA use the PyroMark PCR Kit (cat. No. 978703).
- Before opening the vials, 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.

PyroMark Q24 protocol

- PCR primers 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.
- Sequencing primer should be dissolved in 180 µL Annealing buffer to give a concentration of 10 µM.

PCR Using the PyroMark PCR Kit

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 1. 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.
5. Program the thermal cycler according to Table 2.
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 “Assay and Run Setup”.

Table 1. 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 MgCl2 (final concentration of 1.5 mM).

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	

Assay and Run Setup

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, Figure 1.

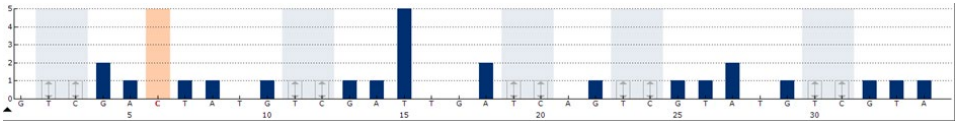

Figure 1. Histogram for the MLH1 assay.

   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 protocol

- PCR primers 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.
- Sequencing primer should be dissolved in 180 µL Advanced Annealing buffer to give a concentration of 10 µM.

PCR Using the PyroMark PCR Kit

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 3. 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.
5. Program the thermal cycler according to Table 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 “Assay and Run Setup”.

Table 3. 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 4. 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	

Assay and Run Setup

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 2.

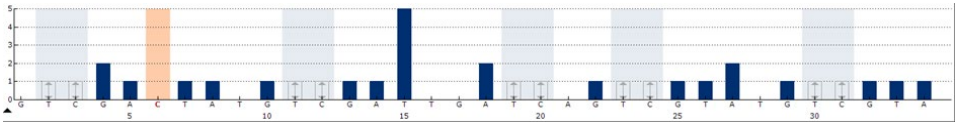

Figure 2. Histogram for the MLH1 assay.

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 Advanced User Manual*.

PyroMark Q48 Autoprep protocol

- PCR primers 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.
- Sequencing primer should be dissolved in 180 µL Advanced Annealing buffer to give a concentration of 10 µM. Dilute the sequencing primer further down to a final concentration of 4 µM.

PCR Using the PyroMark PCR Kit

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 5. 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.
5. Program the thermal cycler according to Table 6.
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 “Assay and Run Setup”.

Table 5. 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	–

Table 6. 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	

Assay and Run Setup

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 3.

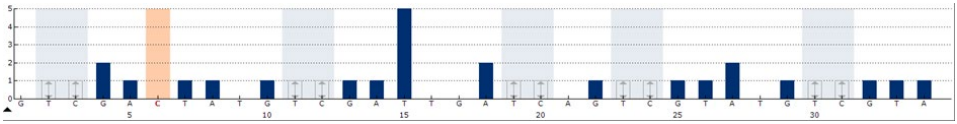

Figure 3. Histogram for the MLH1 assay.

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 Q48 Autoprep User Manual*.

Document Revision History

Date	Changes
07/2024	Initial release.

Scan QR code for *PyroMark Q24 CpG MLH1 Handbook*.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual.

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