

PyroMark[®] Q24 CpG MGMT Assay

The PyroMark Q24 CpG MGMT assay is used to quantify the methylation of CpG sites of the MGMT gene by real-time, sequence-based Pyrosequencing[®]. In this assay the level of methylation is detected for CpG sites in the genomic region chr10:129,467,243-129,467,291 (Reference genome: GRCh38.p14) of the MGMT gene (Ensembl ID: ENSG00000170430). Determination of methylation requires bisulfite conversion of DNA to convert unmethylated cytosines to uracils 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 MGMT kit should be shipped on dry ice and stored at $-20\text{ }^{\circ}\text{C}$ immediately upon arrival. Dissolved Primers should be stored at $-20\text{ }^{\circ}\text{C}$.

Further information

- *PyroMark[®] Q24 CpG MGMT Handbook*: www.qiagen.com/HB-3527
- 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 primer should be dissolved in 120 μL high purity water (Milli-Q® 18.2 $\text{M}\Omega \times \text{cm}$ 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 104 bp.
8. Proceed to “Assay and Run Setup”

Table 1. Reaction composition using PyroMark PCR Master Mix

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 MGMT Assay by selecting **New CpG Assay** in the PyroMark Q24 Software and enter the following sequence in **Sequence to Analyze**:

YGTTTTGYGTTYGAYGTTYGTAGGTTTT

2. **Nucleotide Dispensation Order** – Click **Generate Dispensation Order**. Choose the T at dispensation 26 as a control for bisulfite treatment by left-clicking the orange T. The following dispensation order should be used:

GTCGTTAGTCAGTTCGTATCAGTCGTC^A

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 yellow, Figure 1.

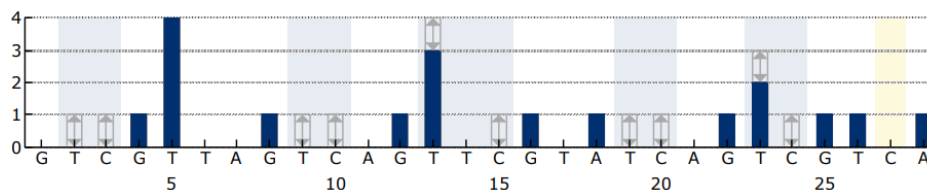


Figure 1. Histogram for the MGMT assay.

The nucleotide addition of T at dispensation 17 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 17, and save the assay.

3. **Run Setup** – Create a new run file by select **New Run**. Setup the plate by adding the MGMT 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 primer should be dissolved in 120 μL high purity water (Milli-Q® 18.2 M Ω \times 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 104 bp.
8. Proceed to “Assay and Run Setup”

Table 3. Reaction composition using PyroMark PCR Master Mix

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 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 MGMT Assay by selecting **New CpG Assay** in the PyroMark Q24 Advanced Software and enter the following sequence in **Sequence Before Bisulfite Treatment (Forward Orientation)**:

CGCTTTGCGTCCCGACGCCCGCA

The software automatically generates the following Sequence to Analyze:

YGTTTTGYGTTTYGAYGTTYGTA

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

GTCGTTAGTCAGTTCTGTATCAGTCTGCT

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.

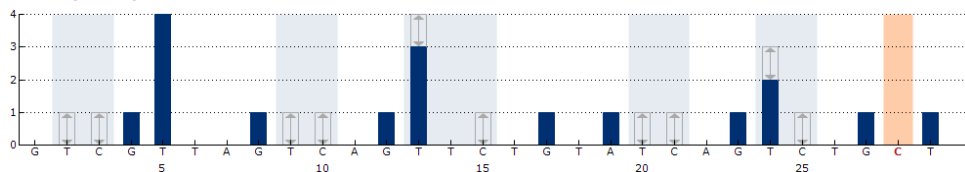


Figure 2. Histogram for the MGMT assay.

The nucleotide addition of T at dispensation 5 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 5, and save the assay.

3. **Run Setup** – Create a new run file by selecting **New Run**. Set up the plate by adding the MGMT 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 primer should be dissolved in 120 μL high purity water (Milli-Q® 18.2 $\text{M}\Omega \times \text{cm}$ 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 104 bp.
8. Proceed to “Assay and Run Setup”

Table 5. Reaction composition using PyroMark PCR Master Mix

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 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

When using PyroMark Q48 Autoprep, the analyzed sequence may be expanded up to 10 CpG sites. This can be done by expanding the sequence before bisulfite treatment (see “Using up to 10 CpG sites”, page 11).

Using up to 5 CpG sites

1. **Sequence to Analyze** – Setup the MGMT assay by selecting **New CPG Assay** in the PyroMark Q48 Autoprep Software and enter the following sequence in **Sequence Before Bisulfite Treatment**:

CGCTTTGCGTCCCGACGCCCGCAG

The software automatically generates the following Sequence to Analyze:

YGT TTTG YG TTT YG AY GTTY GTAG

2. **Nucleotide Dispensation Order** – Click **Generate Dispensation Order**. Choose the T at dispensation 26 as a control bisulfite treatment by left-clicking the orange T. The following dispensation order should be generated and used:

GTCGTTAGTCAGTTCTGTATCAGTCTGCTA

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

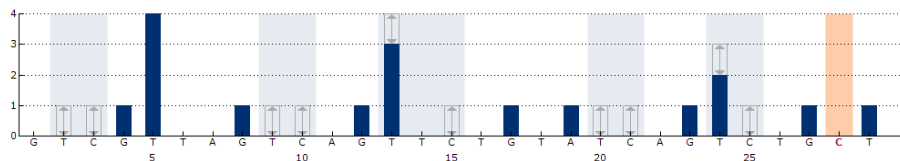


Figure 3. Histogram for the MGMT assay (5 CpG sites).

The nucleotide addition of T at dispensation 5 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 5, and save the assay.

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

Using up to 10 CpG sites

The PyroMark Q48 Autoprep allows for longer Pyrosequencing runs compared to the PyroMark Q24. If you want to analyze up to 10 CpG sites instead of 5, use the following sequences below and adapt it to the desired length if needed.

Due to internal quality criteria, it is possible that the software assesses the additional CpG sites a lower quality, indicated in yellow in the quality bar. Results of these CpG sites are still trustworthy as long as the quality bar does not show a red mark. More details about the quality bar can be found in the instrument user manuals.

Sequence Before Bisulfite Treatment: CGCTTTGCGTCCCGACGCCCGCAGGTCTCGGGTGCACCGTTTGCG

Sequence to Analyze: YGTTTTGYGTTTYGAYGTTYGTAGGTTTTYGGTGYGTATYGTTGYG

Dispensation Order: GTCGTTAGTCAGTTCTGTATCAGTCTGCTATGTTCTAGTCGTAGTCGTATCTGTAGTC

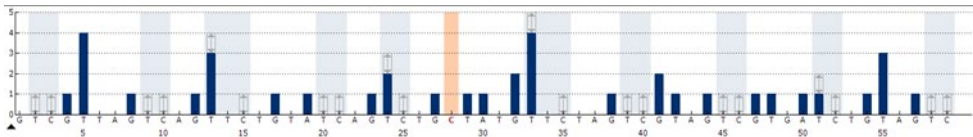


Figure 4. Histogram for the MGMT assay (10 CpG sites).

Document Revision History

Date	Changes
07/2024	Initial release.



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

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