EpiDirect® MGMT

Methylation qPCR Assay

In vitro Diagnostic Assay for Semi-quantitative Analysis of MGMT Promoter Methylation

INSTRUCTIONS FOR USE

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REFERENCE NUMBERS

Ready-to-Use (RTU)
8300 (48 reactions)

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CE IVD

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1 Intended purpose

EpiDirect® MGMT Methylation qPCR Assay is a semi-automated real-time Polymerase Chain Reaction (PCR) assay intended for the direct, semi-quantitative analysis of the methylation status of the human gene of the DNA repair enzyme O6-methylguanine-DNA methyltransferase (*MGMT*). The assay is intended for purified genomic DNA (gDNA) samples that without further pre-treatment can be used with real-time PCR systems for the detection and quantification of the methylation status of the *MGMT* promoter. Samples comprise solid biopsies such as Formalin-Fixed Paraffin-Embedded (FFPE) material and can be prepared using automated platforms or in manual workflows. Methylation of *MGMT* is a prognostic and predictive marker in solid biopsies from glioblastoma patients. EpiDirect® MGMT Methylation qPCR Assay is intended to detect and provide the relative quantity of the amount of methylated versus unmethylated DNA in the *MGMT* promoter.

1.1 Intended user

The assay is intended for use by healthcare professionals or qualified laboratory personnel specifically instructed and trained in the techniques of Real-Time PCR as well as proficient in handling biological samples. Medical interventions based on results from this product requires medical authorisation.

2 Background

Glioblastoma, also known as glioblastoma multiforme (GBM) is the most aggressive form of brain cancer, and around 250,000 persons are diagnosed with glioblastoma worldwide each year[1]. Treatment of the disease involves surgery followed by chemotherapy and/or radiotherapy. The medication temozolomide (TMZ) is often used as chemotherapies, but several studies have shown low efficiency of TMZ in GBM with intact MGMT enzyme. The MGMT enzyme corrects the alkylation made by TMZ, and therefore the TMZ is most effective in case of low to zero activity of the MGMT enzyme. A correlation between hypermethylation of the *MGMT* promoter and enzyme activity has been found, and therefore the methylation status of the *MGMT* promoter is evaluated prior to treatment selection. This is especially relevant for elderly patients who cannot withstand the combination of both radiotherapy and TMZ. Furthermore, better prognosis has been observed in patients with methylated *MGMT* promoter independent of treatment applied. Therefore, the methylation status of the *MGMT* promoter is both a predictive and prognostic marker in glioblastoma[2].

3 Test principle

3.1 Explanation of the assay

EpiDirect® MGMT Methylation qPCR Assay is a duplex assay comprising both HydrolEasy™ probe, EpiPrimer™ and SuPrimer™ technologies for methyl-specific PCR amplification and evaluation of the methylation status of four CpGs in the *MGMT* promotor located on chromosome 10, GRCh38.p13 from 129,467,250 to 129,467,263: **CG**TCC**CG**A**CG**CC**CG**. HydrolEasy™ probe, EpiPrimer™ and SuPrimer technologies are based on modified Intercalating Nucleic Acid (INA®) oligonucleotides containing at least one synthetic DNA analogue (pentabase) comprising a flat heteroaromatic, hydrophobic molecule and a linker. These modifications are inserted into the oligonucleotides at fixed positions during synthesis. The methylation targeting is performed on the FAM channel and an internal reference is targeting a region of the *TBP* gene on the HEX/VIC channel. A heat stable DNA polymerase enzyme is used for amplification. The methylation status of the *MGMT* promoter is quantified based on the ΔCt calculated between the obtained Ct of the *TBP* internal reference and the Ct of the methyl-specific *MGMT* promoter assay.

3.1.1 HydrolEasy™ probes

A HydrolEasy™ probe is similar to a standard hydrolysis probe (also referred to as a TaqMan® probe[3]) labelled with a fluorophore at the 5' end and a quencher at the 3' end, but modified with PentaBase's proprietary technology, giving the probe a significantly improved signal-to-noise ratio, higher specificity, and higher sensitivity compared to conventional hydrolysis probes. HydrolEasy™ probes in the EpiDirect® MGMT Methylation qPCR Assay are labelled with PentaGreen™ (detected on the same channel as FAM) in combination with Green Quencher™, or as PentaYellow™ (detected on the same channel as HEX, VIC®, TET) in combination with Yellow Quencher™.

3.1.2 EpiPrimer™

An EpiPrimer™ is an unique oligonucleotide modified with PentaBase's technology allowing it to bind to methylated DNA with a higher thermal stability compared to unmethylated DNA.

3.1.3 SuPrimers™

SuPrimers™ are standard DNA primers modified with PentaBase's technology. SuPrimers™ can provide increased specificity, sensitivity, and reduce primer-dimer formation.

[1] Gliocure. ' GLIOBLASTOMA' https://www.gliocure.com/en/patients/glioblastoma/ Accessed: 16/05/2022
[2] Hegi, M. E. *et al.* (2005) ' MGMT Gene Silencing and Benefit from Temozolomide in Glioblastoma ', *New England Journal of Medicine*, 352(10), pp. 997–1003. doi: 10.1056/nejmoa043331
[3] Taqman is a registered tradename of Roche Molecular Systems, Inc

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3.2 Product variants

EpiDirect® MGMT Methylation qPCR Assay is supplied as a Ready-to-Use (RTU) product, which is pre-dispensed and only need the addition of a template before analysis.

3.3 Principle of the procedure

Evaluation of the *MGMT* promoter methylation status by EpiDirect® MGMT Methylation qPCR Assay is performed on real-time PCR instruments.

4 Reagents and materials

The materials provided with EpiDirect® MGMT Methylation qPCR Assay can be found in **Table 1**. Materials required, but not provided can be found in **Table 2**. Refer to section 5 for the hazard information for the products.

4.1.1 Storage

Refer to the label for expiry date. Repeated thawing and freezing should be kept to a minimum and should not exceed 9 freeze-thaw cycles.

4.1.2 In-use stability

When in use, the assay components should be returned to the freezer promptly after use, to minimise the time at room temperature and exposure to light. Alternatively, the assay components can be stored at 4°C for up to 5 days prior to use. Used PCR tubes should be disposed following your local guidelines on disposal of biological waste. The reagents included in EpiDirect® MGMT Methylation qPCR Assay are not for reuse.

4.2 Materials provided

Table 1. List of materials provided with the EpiDirect® MGMT Methylation qPCR Assay.

Kit components	Content
EpiDirect® MGMT Methylation qPCR Assay (48 rxn)	Synthetic DNA. Enzymes and buffer for qPCR.
EpiDirect® MGMT Positive control (12 rxn)	25% methylated DNA from cell line.
EpiDirect® MGMT Negative control (12 rxn)	Unmethylated human genomic DNA

4.3 Materials and instruments required but not provided

Materials and instruments required but not provided are listed in **Table 2**. EpiDirect® MGMT Methylation qPCR Assay is designed to run on qPCR platforms with at least two-channels (green and yellow) and has been validated on the CFX96™ System and the CFX Opus 96 Real-Time PCR System (Bio-Rad) as well as the QuantStudio™ 5 Real-Time PCR System (Applied Biosystems™). EpiDirect® MGMT Methylation qPCR Assay was validated using samples purified with BasePurifier™ 32 (PentaBase, ref. no. 715) and QIAamp DNA FFPE Tissue Kit (Qiagen, cat. No. 56404).

Table 2. List of materials and instruments required but not provided.

Materials
Pipette (1-10 µL)
Sterile pipette Tips
Centrifuge for spinning PCR tubes, strips, or plates
Nuclease free water for No Template Control (NTC)
DNA extraction method or instrument
DNA extraction method or instrument (e.g., BasePurifier™ 32, PentaBase A/S, Ref. no. 715)
DNA Extraction kit for FFPE samples (e.g., Nucleic Acid Extraction Kit For FFPE DNA Extraction, Xi'an TianLong Science and Technology Co., Ltd., distributed by PentaBase A/S, Ref. No. T165H)
Real-time qPCR
Real-time PCR instrument: CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Cat. No. 1855196), CFX Opus 96 Real-Time PCR System (Bio-Rad, Cat. No. 12011319) or QuantStudio™ 5 Real-Time PCR System (Applied Biosystems)

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5 Warnings and precautions

- For in vitro diagnostic use.
- Treat all biological specimens as if capable of transmitting infectious agents. All biological specimens should be treated with universal precautions.
- Follow your institution's safety procedures for working with chemicals and handling biological samples.
- Good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary. Wear laboratory coats, laboratory gloves and eye protection when handling biological samples and reagents. Laboratory gloves must be changed between handling different biological samples to avoid contamination of reagents.
- Remove gloves and wash hands thoroughly after handling samples and reagents.
- Do not use EpiDirect® MGMT Methylation qPCR Assay reagents that have expired.
- Do not use damaged EpiDirect® MGMT Methylation qPCR Assay tubes.
- Do not use a EpiDirect® MGMT Methylation qPCR Assay PCR tube that have been dropped while open.
- Do not open the tubes or unseal wells during or after amplification following the completion of the PCR program.
- Consult relevant nucleic acid extraction and real-time qPCR Instrument User Guides for additional warnings, precautions, and procedures to reduce the risk of contamination.
- Be aware of the placement and orientation of the PCR tubes in the PCR machine in relation to how the samples are named in the PCR software.
- Dispose of used EpiDirect® MGMT Methylation qPCR Assay tubes, pipette tips and specimen tubes according to local, state, and federal regulations for biological material.
- Due to the high sensitivity of the assays, contamination of the work area with previous samples might cause false results. Therefore, use extreme caution not to contaminate reagents and handle samples according to standard laboratory practice.
- The reagents should not be diluted to a lower concentration than stated in the protocol. This may affect the performance of the assay.
- Do not substitute the reagents with others as it may affect the performance of the assay.
- Inadequate or inappropriate sample collection, storage, transport, and purification may yield incorrect or invalid results.
- Different platforms (PCR cyclers) might influence the results. Thus, carefully read this IFU and use a validated instrument.

6 Sample handling

6.1 Sample collection

Specimens should be human genomic DNA extracted from Formalin-Fixed Paraffin-Embedded (FFPE) tumour sections or similar. It is recommended that FFPE samples are collected, transported, processed, and stored according to ISO 20166-3:2018[4] to ensure optimal DNA quality.

6.2 Sample purification

Extraction of genomic DNA from FFPE samples should be performed using genomic DNA extraction kits and/or procedures specially designed for handling of FFPE samples according to the manufacturer´s instructions, including steps for deparaffinisation and sample digestion to remove PCR inhibitors embedded in the sample. Refer to **Table 2** for validated purifications methods. It is recommended to evaluate DNA integrity and amplifiability by PCR-based methods according to ISO 20166-3:2018.

6.3 Positive and Negative Controls

At least one Positive and one Negative Control should be included in each EpiDirect® MGMT Methylation qPCR Assay real-time qPCR run.

The Positive Control contains DNA from a methylated cell-line mixed with unmethylated human genomic DNA to obtain a methylation percentage of approximately 25%. The Negative Control contains unmethylated human genomic DNA. The concentration of the control samples is 1 ng/µL.

[4] Molecular in vitro diagnostic examinations — Specifications for pre-examination processes for formalin-fixed and paraffin-embedded (FFPE) tissue — Part 3: Isolated DNA

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7 Procedure

1. Spin down the PCR strips (10 seconds at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the strips.
2. Add 5 µL of extracted DNA (0.1-10 ng/µL) from each sample to the PCR tubes.
3. For each PCR run add 5 µL of positive control and 5 µL of negative control to two separate PCR tubes.
4. It is optional to add 5 µL of nuclease free water (no template control, NTC) to a PCR tube to test for contamination.
5. Seal all PCR tubes.
6. Recommended step: Briefly vortex strips (2-3 sec.) to enhance elimination of air bubbles.
7. Spin down the PCR tubes (1-2 minutes at 4000-5000 rpm) to ensure that all reagents are collected at the bottom of the tubes and to eliminate air bubbles.
8. Place the PCR tubes in the real-time PCR instrument and run the real-time qPCR program (**Table 3**).

7.1 Real-time PCR program

Table 3. Real-time qPCR program for EpiDirect® MGMT Methylation qPCR Assay.

Protocol	Temperature [°C]	Time [sec]	Cycles	Channel
Stage 1				
Hold	95	120	1	
Stage 2 (1-5)				
Amplification	100	60	5	
	76	60		
Stage 3 (6-40)				
Amplification	98	10	40	
	76	60		FAM + HEX/VIC

8 Data Analysis

8.1 Bio-Rad CFX Opus 96 Real-Time PCR System (CFX)

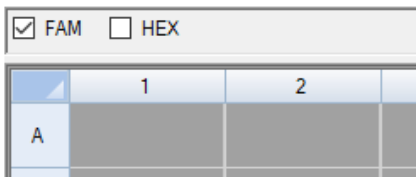
8.1.1 Baseline and threshold settings

The threshold should be adjusted according to the positive control sample and should be adjusted to 10% of the maximum RFU value on both the FAM and HEX channel.

To adjust the threshold setting on the Bio-Rad CFX Opus 96 Real-Time PCR System:

1. Select one fluorophore at a time (**Figure 1**)

Figure 1 Selecting setting for the fluorophore. Shown is the selecting of FAM.



2. Go to “Settings” and press “Baseline threshold…”
3. Select “User Defined:” under “Single Threshold” and adjust to 10% of the maximum RFU of the positive control sample.
    a. E.g., if the maximum RFU on the FAM channel is 22,000, the threshold should be set to 2,200 RFU (**Figure 2**).
4. Repeat for the HEX channel.
    a. E.g. if the maximum RFU on HEX channel is 5,000, the threshold should be set to 500 RFU.

Figure 2. Adjusting the threshold to 10% of the maximum.

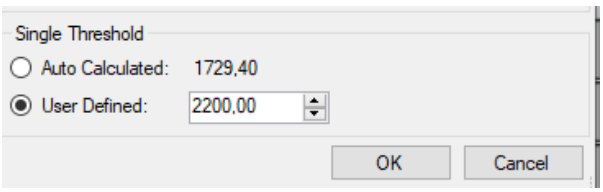


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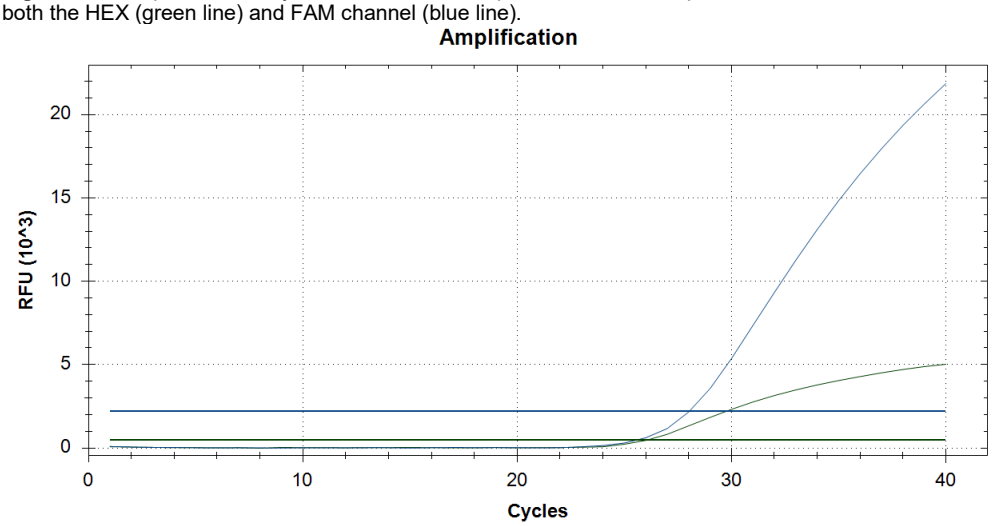
Figure 3. Example of correctly set threshold for the positive control sample. The threshold is set to 10% of the maximum RFU value on both the HEX (green line) and FAM channel (blue line).



In case of air bubbles in the PCR tubes, the baselining should be adjusted to start after the air bubbles are out of the tubes and before the exponential phase of the PCR. In the case of air bubbles, baselining can be set between cycle 10-20. Adjust the baselining in the same pop-up window when setting the threshold.

To adjust the baselining on CFX Opus 96 Real-Time PCR System:

a) Select one fluorophore at the time.
b) Go to "Settings" → "Baseline threshold…"
c) Select "User Defined" under Baseline Cycles. Select all rows and adjust "All Selected Rows:" from "Begin:" to "End:".

8.1.2 Run validation

Before interpreting the result, it should be validated that the PCR run has been performed correctly. The run validation criteria are summarised in **Table 4**.

Table 4. Acceptance criteria for run validation.

Metric	Acceptance criteria
Sample Ct value (HEX channel)	25-28
Positive Control ΔCt	1.6-2.4 (corresponding to 17.4-32.9% methylation)
Negative Control ΔCt	>4.9

8.1.3 Interpretation of results

To determine the methylation frequency, a ΔCt value is calculated and is defined as the difference between the Ct value of the FAM channel subtracted the Ct value of the HEX channel:

$$\Delta Ct = Ct_{FAM} - Ct_{HEX} \qquad (1)$$

Calculate the ΔCt (Equation 1). According to the ΔCt, the samples can be grouped into the categories shown in **Table 5**. To get precise estimation of the percentage of the methylation, the ΔCt can be put into Equation 2:

Table 5. Correlation between ΔCt and approximate % methylation.

ΔCt	Approx. methylation frequency
≤1	50-100%
>1-1.9≥	25-50%
>1.9-3.0≥	10-25%
>3.0-4.9≥	3-10%
>4.9	Unmethylated

$$\%methylation = 10^{\frac{\Delta Ct-6}{-2.9}} \qquad (2)$$

If the sample produces a valid signal in the HEX channel, but no signal in the FAM channel, the Ct of FAM should be set to 40, and the ΔCt can then be calculated.

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8.2 QuantStudio™ 5 Real-Time PCR System (QS5)

8.2.1 Baseline and threshold settings

The threshold should be adjusted according to the positive control sample and should be adjusted to 10% of the maximum RFU value on both the FAM (green) and VIC (yellow) channel. To adjust the threshold setting on the QuantStudio™ 5 Real-Time PCR System (QS5):

1. Select one target at a time **(Figure 4)**.
2. Press on the eye and change "Graph Type" to Linear.
3. Unselect the "Auto" setting of the "Threshold" and adjust the threshold to 10% of the maximum RFU of the positive control sample (**Figure 5)**
    a. E.g., if the maximum RFU on the FAM channel is 1,750,000, the threshold should be set to 175,000 RFU.
    b. If the maximum RFU on VIC channel is 250,000, the threshold should be set to 25,000 RFU.
4. Repeat for the other target.
5. Finish by pressing "Analyze" in the top right corner.

Figure 4. Selecting the target. The targets in the example are "Methyl" (FAM channel) and "IC" (VIC).

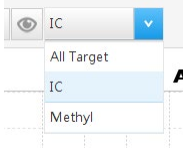
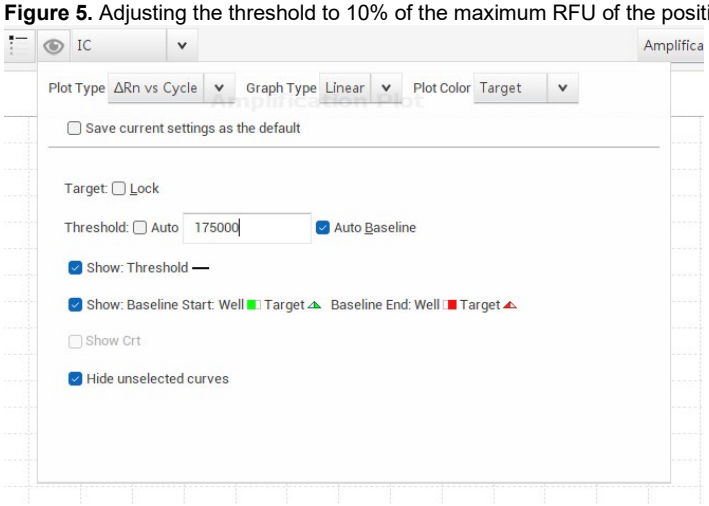


Figure 5. Adjusting the threshold to 10% of the maximum RFU of the positive control.

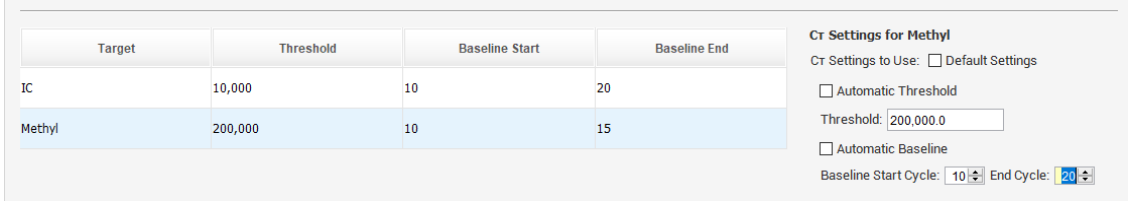


In case of air bubbles in the PCR tubes, the baselining should be adjusted to start after the air bubbles are out of the tubes and before the exponential phase of the PCR. In the case of air bubbles, baselining can be set between cycle 10-20.

To adjust the baselining on the QuantStudio™ 5 Real-Time PCR System (**Figure 6**)

6. Adjust the baselining under "Analysis" and "Analysis Settings…"
7. Press on the target for baseline adjustment and unselect "Automatic Baseline".
8. Change the "Baseline Start Cycle" and "End Cycle".

Figure 6. Adjusting the baseline manually.



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8.2.2 Run validation

Before performing the result interpretation, it should be validated that the PCR run has been performed correctly. The run validation criteria are summarised in **Table 6**.

Table 6. Acceptance criteria for run validation.

Metric	Acceptance criteria
Sample Ct value (VIC channel)	21.0 – 28.0
Positive Control ΔCt	2.1-2.9 (corresponding to 31.6-17.8% methylation)
Negative Control ΔCt	>5.8

8.2.3 Interpretation of results

To determine the methylation frequency, a ΔCt value is calculated and is defined as the difference between the Ct value of the FAM channel subtracted the Ct value of the VIC channel:

$$\Delta Ct = Ct_{FAM} - Ct_{VIC} \qquad (3)$$

Calculate the ΔCt (Equation 3). According to the ΔCt, the samples can be grouped into the categories shown in **Table 7**. To get precise estimation of the percentage of the methylation, the ΔCt can be put into Equation 4:

Table 7. Correlation between ΔCt and approximate % methylation.

ΔCt	Approximate methylation frequency
≤1.5	50-100%
>1.5-2.4≥	25-50%
≥2.4-3.7≥	10-25%
>3.7-4.7≥	5-10%
>5.8	Unmethylated

$$\%methylation = 10^{\frac{\Delta Ct - 6.9}{-3.2}} \qquad (4)$$

If the sample produces a valid signal in the VIC channel, but no signal in the FAM channel, the Ct of FAM should be set to 40, and the ΔCt can then be calculated.

8.3 Invalid samples

8.3.1 Invalid positive control signal

In case of lack of positive control signal (no Ct value) or invalid positive control signal (CFX 1.6 > ΔCt > 2.4, QS5 2.0 > ΔCt > 2.9), please check that the threshold for the FAM and HEX/VIC channels are set to 10% of the maximum RFU of the positive control sample and make sure that the qPCR program has been defined correctly. Repeat the qPCR run. If the positive control signal is still absent for either the FAM or HEX/VIC channels or is invalid in the re-run, the results are invalid and should not be used. The positive control reagents might be degraded or the EpiDirect® MGMT Methylation qPCR Assay not functional. If you cannot locate the root cause of the problem, please contact PentaBase A/S or your local distributor for support.

8.3.2 Invalid sample signal

If the Ct value on the HEX/VIC channel is below 22.8 on the CFX Opus 96 Real-Time PCR System or below 21 on the QS5 instrument, the sample should be diluted further. If the Ct value on the HEX/VIC channel is above 29.6 (CFX) or above 28 (QS5), use a higher concentration of the sample. If no signal occurs in the FAM or HEX/VIC channel of a sample, make sure that the threshold for the FAM and HEX/VIC channels are set to 10% of the maximum RFU of the positive control sample and make sure that the qPCR program has been defined correctly and repeat the qPCR run. If no signal occurs in the re-run, re-validate the DNA concentration of the sample and repeat the qPCR run with a higher concentration of the sample. If you cannot locate the root cause of the problem, please contact PentaBase A/S or your local distributor for support.

8.3.3 Signal in NTC

Signal in the NTC indicates contamination, which may influence the results. Data should not be used if the NTC is positive. Find the cause of contamination by checking or replacing all potential sources of the contamination such as pipettes and instruments and re-run the samples with a new NTC. If the contamination cannot be located contact PentaBase A/S or your local distributor for support.

9 Performance evaluation

The performance evaluation was performed on the Bio-Rad CFX Opus 96 Real-Time PCR System, and the LOD and LOB study was repeated on the Applied Biosystems QuantStudio™ 5 Real-Time PCR System.

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9.1 Analytical sensitivity

9.1.1 Limit of Blank

Limit of blank (LOB) was determined to be ΔCt = 5.0 with 99.5% confidence on the CFX Opus 96 Real-Time PCR System. This was done using 20 replicates of unmethylated human genomic DNA extracted from whole blood in a concentration of 1 ng/µL. The limit of blank was validated by 20 replicates of DNA extracted from an unmethylated FFPE patient sample.

LOB was determined to be ΔCt = 6.0 with 99.5% confidence level on the QuantStudio™ 5 Real-Time PCR System.

9.1.2 Limit of Detection and Limit of Quantification

The limit of detection (LOD) was determined to be 3% methylation with 95% confidence level on the CFX Opus 96 Real-Time PCR System. The limit of detection was evaluated using 20 replicates of DNA from an enzymatic methylated cell line (HCT116 DKO) diluted to 3% methylation in a background of unmethylated human genomic DNA. This was also determined to be the limit of quantification (LOQ).
The LOD was validated using an unmethylated purified FFPE patient sample spiked with methylated DNA from a 100% methylated cell line to obtain 3% methylation. 19 out of 20 replicates were detected by EpiDirect® MGMT Methylation qPCR Assay.

The LOD was determined to be 1.5% methylation with 95% confidence level on the QuantStudio™ 5 Real-Time PCR System. The LOQ was found to be 5% methylation.

NB. There might be difference on LOD on different PCR instruments. However, this should not influence the clinical cutoffs which has been shown to variate from 3%-30% using the golden standard[5].

9.1.3 Linear dynamic range

The linear dynamic range was evaluated for sample concentrations between 0.1 and 10 ng/µL using a 25% methylated sample as well as an unmethylated sample. The 25% methylated sample was made by mixing enzymatic methylated cell line HCT116 DKO (ZYMO Research, Cat #D5014-2) cell line with unmethylated human genomic DNA. For the 25% methylated sample, the PCR efficiency was 97.2% on both the FAM (R^2 = 0.993) and HEX channel (R^2 = 0.989). For the unmethylated sample, the PCR efficiency was 81.0% on the FAM channel (R^2 = 0.973) and 94.9% on the HEX channel (R^2 = 0.988). The assay shows linearity from 100% to 2.5% methylated DNA, on the CFX Opus 96 Real-Time PCR System, using a sample concentration of 1 ng/µL with a R^2 of 0.976. The linear dynamic range on the QuantStudio™ 5 Real-Time PCR System was within 100% to 5% with a R^2 of 0.985, using a sample concentration of 1 ng/µL.

9.2 Analytical precision

9.2.1 Trueness

The trueness was evaluated using DNA from an enzymatic methylated cell line HCT116 DKO (ZYMO Research, Cat #D5014-2) cell line diluted in a background of human genomic DNA on the CFX Opus 96 Real-Time PCR System. The limit of agreement (LOA) can be found in **Table 8**.

Table 8. Trueness of EpiDirect® MGMT Methylation qPCR Assay.

Sample description	Overall bias	Standard deviation	Upper LOA	Lower LOA
5x 25% methylated DNA 10 ng/µL	-1.1%	3.18%	5.1%	-7.4%
5x 25% methylated DNA 1 ng/µL				
5x 25% methylated DNA 0.1 ng/µL				
20x 3% methylated DNA 1 ng/µL				

9.3 Specificity

No genetic variations were found in the primer binding regions by analysis in UCSC database[6]. The primers were found to be 100% specific towards their target regions by blasting in NCBI Primer-BLAST[7] and no cross reactivity was found between the two primer sets.

9.4 Clinical evaluation

EpiDirect® MGMT Methylation qPCR Assay (EpiDirect® MGMT) was validated using a CFX Opus 96 Real-time PCR Instrument and a cohort of 34 FFPE samples from brain tumours (**Table 9**). The samples were previously analysed with bisulfite conversion followed by methyl-specific endpoint PCR (MSP) and evaluated by gel electrophoresis. The samples were diluted to approximately 2 ng/µL and evaluated with EpiDirect® MGMT Methylation qPCR Assay. The methylation agreement was 8/9 or 88.9% (CI 95: 56.5-98.0%) and unmethylated agreement was 20/25 or 80% (CI 95: 60.9-91.1%. The samples found to be methylated by EpiDirect® MGMT Methylation qPCR Assay but unmethylated by MSP had an estimated

[5] https://doi.org/10.1093%2Fnop%2Fnpz039
[6] https://genome.ucsc.edu/
[7] https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi

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methylation percentage between 2.8% and 6.5% based on EpiDirect® MGMT Methylation qPCR Assay ΔCt values. No clinical cut-off was set for the evaluation of this cohort.

Table 9. 2x2 contingency table for EpiDirect® MGMT and methyl-specific endpoint PCR (MSP).

Sample status	MSP MGMT Methylated	MSP MGMT Unmethylated	Total
EpiDirect® MGMT Methylated	**8**	5	13
EpiDirect® MGMT Unmethylated	1	**20**	21
Total	9	25	34

10 Limitations

- Performance of EpiDirect® MGMT Methylation qPCR Assay has only been tested on FFPE specimens from glioblastoma tumours.
- Incorrect collection, storage, DNA extraction, transportation or handling of the sample could cause false test results due to low amount or poor quality of gDNA or the presence of PCR inhibitors in the sample.
- EpiDirect® MGMT Methylation qPCR Assay is validated for use with 0.5-50 ng of DNA per reaction (0.1-10 ng/µL). Using DNA input amounts lower or higher than this may lead to incorrect test results.
- A negative test result (no methylation) does not exclude the presence of methylated DNA at levels below the detection limit of the assay.
- Rare mutations within the genomic DNA regions covered by the oligonucleotides used in the EpiDirect® MGMT Methylation qPCR Assay may impair the detection of methylated DNA.
- EpiDirect® MGMT Methylation qPCR Assay is a semi-quantitative test that will only provide an estimate of the methylation frequency.
- EpiDirect® MGMT Methylation qPCR Assay has only been validated on the CFX96/384™ (Opus) PCR System and QuantStudio™ 5 Real-Time PCR System (Applied Biosystems).
- The diagnostic sensitivity and specificity of EpiDirect® MGMT Methylation qPCR Assay have only been evaluated on the CFX Opus 96 Real-Time PCR System

11 Symbols

The following symbols are used in labelling of EpiDirect® MGMT products.


Date/country of manufacture


Use-by date


Contains sufficient for <*n*>


Temperature limit


In vitro diagnostic medical device


Do not reuse


Manufacturer


Consult electronic available instructions for use


Indicates that this device is in conformity with the essential health and safety requirements set out in the REGULATION (EU) 2017/746 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 5 April 2017 on in vitro diagnostic medical devices and repealing Directive 98/79/EC and Commission Decision 2010/227/EU.

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Manufacturer

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For technical assistance please contact your local distributor or PentaBase A/S. A complete list of distributors is available at www.pentabase.com.

NOTICE TO USERS: Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the patient is established.