



MethylDetect DNA Methylation Assay Kit

Protocol

For 100, 500 and 2500 reactions

Store at 2-8 °C

Date: October 2017. V.1

1. General Information

The performance of each MethylDetect DNA Methylation Assay Kit has been tested on the LightCycler® 480 High Resolution Melting platform (product number: 05015278001), using LightCycler® 480 High Resolution Melting Master (product number: 04909631001) PCR reagents in 96 well plates.

The use of this platform is recommended in combination with the MethylDetect DNA Methylation Assay Kit.

Other RT-PCR systems capable of performing high resolution melting (HRM) can be used following further optimization of the experimental conditions.

1.1. Contents:

| Vial | Label | Content |
|------|---------------------|----------------|
| 1 | Primer Mix | 1 vial, 120 µl |
| 2 | Positive Control | 1 vial, 100 µl |
| 3 | Calibration Control | 1vial, 100 µl |
| 4 | Negative Control | 1vial, 100 µl |

1.2. Storage conditions:

The MethylDetect DNA Methylation Assay Kit is shipped at room temperature.

When stored at 2-8°C the reagents are stable until the expiration date printed on the label.

For storage conditions of the LightCycler® 480 High Resolution Master Mix, the associated protocol can be consulted for further details¹.

1.3. Additional equipment and reagents required:

1. High Resolution Master Mix (containing Taq polymerase, reaction buffer, dNTP mix, HRM dye, MgCl₂, and water, PCR grade).
2. An instrument combining Real-Time PCR with high resolution melting capability.
3. 96 well plates or reaction tubes compatible with the Real-Time PCR and HRM instrument.
4. Nuclease-free, aerosol-resistant pipette tips.
5. Pipettes with disposable, positive-displacement tips.
6. Sterile reaction tubes for preparation of master mix.
7. *Optional:* Standard swinging-bucket centrifuge containing a rotor for multiwell plates.

1.4. Application

The MethylDetect DNA Methylation Assay Kit is designed and optimized for detection of DNA methylation status of the target gene promoter region in human bisulfite converted genomic DNA. Methyl Detect supplies three controls with each kit to ensure optimal performance of the assay. DNA: 1. methylation positive control, 2. assay calibration control, and 3. methylation negative control.

1.5 Preparation time

Assay time

The cycling program for the MethylDetect DNA Methylation Assay Kit includes a 10 min pre-incubation and 50 cycles of amplification followed by high resolution melting. The assay will take about 90-120 min when performed using the LightCycler® 480 System.

2. Protocols

2.1. Before you begin

Sample materials

As a general rule, when using PCR for methylation studies, all DNA samples have to be bisulfite converted, to ensure preservation of the methylated cytosines in the template¹. The changes to the double stranded DNA sequence after bisulfite conversion, in which the cytosines in CpG dinucleotides are methylated, is illustrated in figure 1:

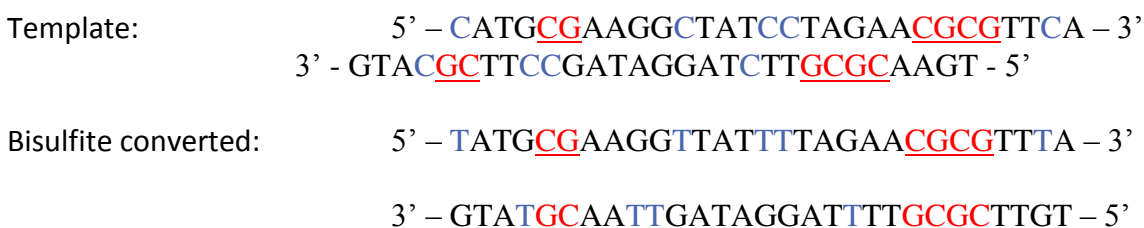


Figure 1. The preserved methylated cytosines, which are part of a CpG dinucleotide, are underlined and in red. The unmethylated cytosines are converted to uracil, and further substituted with thymine during the PCR, and are illustrated in blue. After bisulfite conversion the DNA strands are no longer complementary, and will appear single-stranded.

Following bisulfite treatment the methylated DNA sequence is different from the unmethylated DNA sequence, since unmethylated cytosines are converted to uracil, and methylated cytosines remain unchanged. After PCR, the products will have different melting properties, and the resulting profile after HRM enables discrimination between methylated and unmethylated templates, respectively (figure 2).

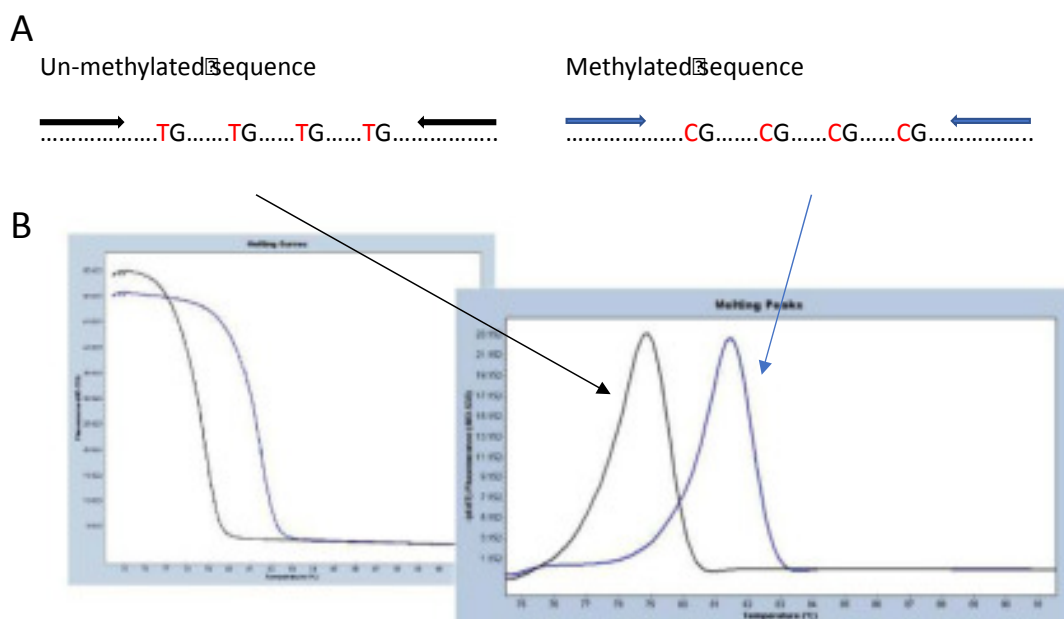


Figure 2. Schematic illustration of the principle behind HRM analysis. A) the difference in a DNA sequence after bisulfite conversion of a methylated and an unmethylated genomic region. B) the difference in melting properties of the PCR products from the methylated (blue) and the unmethylated (black) templates.

Sample DNA

Use 50-100 ng of bisulfite modified DNA per reaction. This is a theoretical concentration based on the DNA input for bisulfite conversion and the elution volume. Commercial kits are available for bisulfite conversion of the sample DNA. In the current version of the MethylDetect DNA Methylation Assay Kit, we recommend the use of bisulfite conversion kits from Zymo Research to process DNA samples prior to methylation analyses.

! Follow the instructions from the bisulfite conversion kit for storage of the DNA after treatment ².

The quality of the DNA should be suitable for PCR in terms of concentration, purity and absence of PCR inhibitors. Use of the same DNA extraction procedure for all samples may eliminate any subtle differences in the high-resolution melting results. To ensure sufficient quality of the DNA prior to bisulfite conversion, agarose gel electrophoresis or analysis by a Bioanalyzer can be used to assess the DNA integrity, and a Qubit Fluorometer is recommended for measuring the DNA concentration.

Assay calibration controls

A positive, an assay calibration, and a negative control are supplied with the MethylDetect DNA Methylation Assay Kit, and are ready for use. The assay calibration control is included to ensure the assay sensitivity to detect methylation of 1%.

! The positive, the assay calibration, and the negative controls are not to be bisulfite converted.

The controls are each applied in triplicates to all multiwell plates.

Negative Control Reaction

A No Template Control (NTC) should always be included in each analysis. The NTC contains the same reagents as the reactions for analyses, except that the DNA sample is replaced with the same amount of PCR grade water. The NTC should be present at each multiwell plate in triplicates.

Primers

A primer set is supplied within the MethylDetect DNA Methylation Assay Kit, and the PCR assay conditions are given in the Protocol table.

Mg⁺ Concentration

The optimum MgCl₂ concentration is essential to enhance amplification after bisulfite modification and thereby highly important for the high-resolution melting result. The recommended MgCl₂ concentration for the Methyl Detect DNA Methylation Assay Kit reaction is provided in table 1.

2.2. Preparation of the PCR reaction

The MethylDetect DNA Methylation Assay Kit protocol is calibrated using the LightCycler® 480 High Resolution Melting Master and the associated protocol can be consulted for further details [1](#).

Follow the procedure below in the given order to prepare one 20 µl standard reaction.

1. Thaw the solutions and spin all tubes briefly in a micro-centrifuge before opening, to ensure that the content is collected at the bottom of the tube.

-store all reagents on ice.

2. Prepare the PCR mix for one 20 µl reaction by adding the following components in the order listed below and keep it on ice.

Table 1.

| Component | Volume |
|------------------------------|--------|
| HRM Master 2 × conc. | 10 µl |
| Primer mix | 1.0 µl |
| MgCl ₂ (25 mM) | 2.4 µl |
| H ₂ O (PCR grade) | 0.6 µl |

Multiple Reactions.

To prepare the PCR mix for multiple reactions, multiply the volume for each component with the number of reactions, including the standard control samples (positive, assay calibration, and negative control DNA) and the NTC in triplicates, adding extra reactions to compensate for loss of reagents during pipetting (we suggest for a 96-well plate prepare 100 reactions).

! For plate setup see figure 3.

Example:

To prepare a 96-well plate, in which all samples are analysed in triplicates: use 28 test samples (84 wells) + three different standards (nine wells) + one NTC (three wells) = 96 wells.

3. Mix the reagents carefully by pipetting up and down and spin briefly. **Do not vortex.**

Pipette 14 μ l PCR mix into each well, including the wells to contain the positive, the assay calibration, the negative, and the no template controls.

4. Add 6 μ l bisulfite treated DNA, corresponding to a theoretical calculated value of 50-100 ng DNA. For optimal performance, the amount of template was tested in the range of 50-100 ng. Lower amount of template can be used however, we recommend that the assay is optimized to the specific DNA concentration before processing the test samples.

! For each multiwell plate, add 6 μ l of each standard control DNA provided in the Methyl Detect DNA Methylation Assay Kit (positive, assay calibration, and negative), in triplicates.

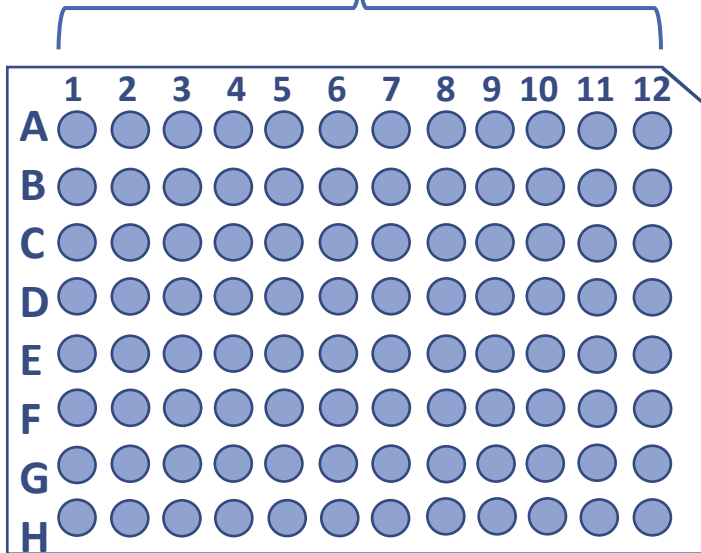
Seal the multiwell plate with an appropriate sealing foil.

5. *Optional*; Place the multiwell plate in a standard swinging-bucket centrifuge and spin for 2 minutes at 1000 \times g.
6. Place the multiwell plate in the instrument and start the PCR-HRM program.

Step 1



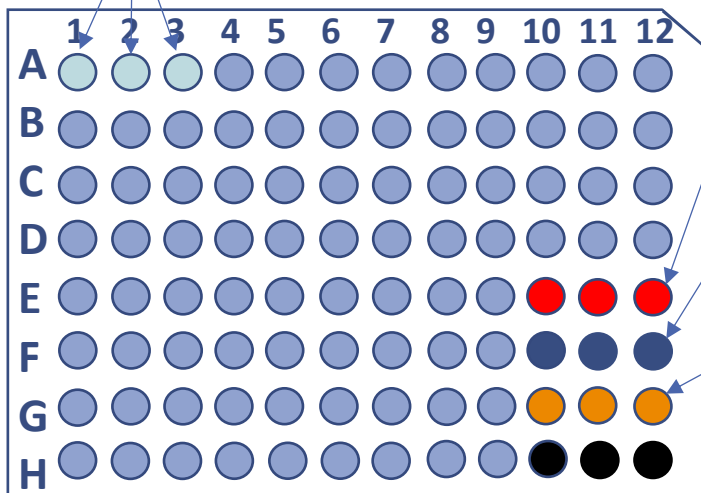
Distribute the PCR mix (table 1) in all wells



Step 2



DNA sample no 1
Distributed in duplicates
(or triplicates)



Methylation
Positive Control
In triplicates

Assay Calibration Control
In triplicates

Methylation
Negative Control
In triplicates

No template control (NTC)
(Black)

Figure 3. Plate setup.

2.3. PCR and HRM program

The program below is suitable for the LightCycler® 480 System, consult the protocol ¹

| Program | Cycles | Temperature (°C) | Hold (sec) | Ramp Rate (°C/sec) | Acquisitions (per °C) |
|-------------------------|--------|------------------|------------|--------------------|-----------------------|
| Pre-Incubation | 1 | 95 | 600 | | |
| Amplification | 50 | 95 | 15 | 4.4 | None |
| | | 58* | 10 | 2.2 | None |
| | | 72 | 15 | 4.4 | Single |
| High Resolution Melting | | 95 | 15 | 4.4 | None |
| | | 60 | 60 | 2.2 | None |
| | | 95 | Continuous | 0.01 | 50 |

* The optimal annealing temperature for each Methyl Detect DNA Methylation Assay can be found in the gene specific data for each assay kit in the product catalog at www.methyldetect.com.

3. Results

To see examples of the results you can obtain with the MethylDetect DNA Methylation Assay Kit, please go to www.methyldetect.com and view the gene specific data for each assay in the product catalog.

4. Troubleshooting

The MethylDetect DNA Methylation Assay Kit protocol was calibrated using the LightCycler® 480 High Resolution Melting Master and the protocol specific for this kit can be consulted for troubleshooting ².

5. The principles behind the MS-HRM analysis method

Methylation-Sensitive High-Resolution Melting (MS-HRM) is a high-throughput technology for highly sensitive DNA methylation analysis of single loci ^{2,3}.

The technology utilizes the difference in melting properties of the PCR product amplified from methylated and unmethylated DNA strands after bisulfite conversion. The inclusion of standard DNA with known DNA methylation status ensures a highly sensitive read-out of the methylation of the test DNA.

MS-HRM was shown to differentiate between methylated, un-methylated, and heterogeneous methylated templates, which have clearly distinguishable profiles after High-Resolution Melting (HRM) ^{4 5}.

References

1. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* 1994;22(15):2990-2997.
2. Wojdacz TK, Dobrovic A, Hansen LL. Methylation-sensitive high-resolution melting. *Nat Protoc.* 2008;3(12):1903-1908.
3. Wojdacz TK, Dobrovic A. Methylation-sensitive high resolution melting (MS-HRM): a new approach for sensitive and high-throughput assessment of methylation. *Nucleic Acids Res.* 2007;35(6):e41.
4. Wojdacz TK, Windelov JA, Thestrup BB, Damsgaard TE, Overgaard J, Hansen LL. Heterogeneous and low-level methylation of novel biomarker candidates for breast cancer clinical management. *Cancer Research.* 2014;74(19).
5. Bock C, Halbritter F, Carmona FJ, et al. Quantitative comparison of DNA methylation assays for biomarker development and clinical applications. *Nature Biotechnology.* 2016;34(7):726-+.

6. Supplementary Information

Assay Specific Information

Results and annealing temperatures: examples of the results from a MethylDetect DNA Methylation Assay Kit can be found in the product catalog for each kit, along with the annealing temperature.

Ordering Information

MethylDetect offers a number of DNA methylation detection kits targeting specific genomic regions. For a complete overview of the products please visit www.MethylDetect.com

License Disclaimer

For patent licence limitations for individual products please refer to www.MethylDetect.com

Regulatory Disclaimer

For Life Science research only. Not for use in diagnostic procedures.

Safety Data Sheet

Please follow the instructions in the Safety Data Sheet (SDS) at www.MethylDetect.com

Contact and Support: www.MethylDetect.com; email: info@methyldetect.com