August 2011

EpiTect® MethyLight PCR Handbook

EpiTect MethyLight PCR Kit

EpiTect MethyLight PCR + ROX™ Vial Kit

For quantitative methylation analysis using sequence-specific probe-based real-time PCR

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Contents

Kit Contents

EpiTect MethyLight PCR Kit	**(200)**	**(1000)**
Cat. no.	**59436**	**59438**
Number of reactions (50 µl)	**200**	**1000**
2x EpiTect MethyLight Master Mix, containing: ■ HotStarTaq® *Plus* DNA Polymerase ■ EpiTect Probe PCR Buffer ■ dNTP mix (dATP, dCTP, dGTP, dTTP) ■ ROX passive reference dye	3 x 1.7 ml	25 ml
RNase-Free Water	2 x 2 ml	20 ml
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EpiTect MethyLight PCR + ROX Vial Kit	**(200)**	**(1000)**
Cat. no.	**59496**	**59498**
Number of reactions (50 µl)	**200**	**1000**
2x EpiTect MethyLight Master Mix (w/o ROX), containing: ■ HotStarTaq *Plus* DNA Polymerase ■ EpiTect Probe PCR Buffer ■ dNTP mix (dATP, dCTP, dGTP, dTTP)	3 x 1.7 ml	25 ml
50x ROX Dye Solution	210 µl	1.05 ml
RNase-Free Water	2 x 2 ml	20 ml
Handbook	1	1

Shipping and Storage

EpiTect MethyLight PCR Kits are shipped on dry ice. The kits should be stored immediately upon receipt at –20°C in a constant-temperature freezer and protected from light. When stored under these conditions and handled correctly, this product can be stored at least until the expiration date (see the inside of the kit lid) without showing any reduction in performance.

Product Use Limitations

The EpiTect MethyLight PCR Kits are intended for molecular biological applications. These products are 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.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN® will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the EpiTect MethyLight PCR Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

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

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Product Specifications

EpiTect MethyLight PCR Kit

2x EpiTect MethyLight Master Mix contains:

HotStarTaq *Plus* DNA Polymerase:	HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into *E. coli*. (Deoxynucleoside-triphosphate: DNA deoxynucleotidyl-transferase, EC 2.7.7.7). The enzyme is activated by a 5-minute, 95°C incubation step.
EpiTect Probe PCR Buffer	Novel PCR buffer for highly sensitive methylation quantification of bisulfite converted DNA targets; includes multiplex-PCR–enabling Factor MP.
dNTP mix:	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality.
ROX passive reference dye:	Optimized concentration of fluorescent dye for normalization of fluorescent signals on instruments from Applied Biosystems (models 7000, 7300, 7700, 7900HT, StepOne™, and StepOne Plus™, but not Applied Biosystems® 7500 Real-Time PCR Systems).
RNase-free water:	Ultrapure quality, PCR-grade.

EpiTect MethyLight PCR + ROX Vial Kit

2x EpiTect MethyLight PCR Master Mix (w/o ROX) contains:

HotStarTaq *Plus* DNA Polymerase:	HotStarTaq *Plus* DNA Polymerase is a modified form of a recombinant 94 kDa DNA polymerase, originally isolated from *Thermus aquaticus,* cloned into *E. coli.* (Deoxynucleoside-triphosphate: DNA deoxynucleotidyl-transferase, EC 2.7.7.7). The enzyme is activated by a 5-minute, 95°C incubation step.
EpiTect Probe PCR Buffer:	Novel PCR buffer for highly sensitive methylation quantification of bisulfite converted DNA targets; includes multiplex-PCR–enabling Factor MP
dNTP mix:	Contains dATP, dCTP, dGTP, and dTTP of ultrapure quality
50x ROX Dye Solution:	Separate tube of passive reference dye for normalization of fluorescent signals on Applied Biosystems 7500 Real-Time PCR Systems and, optionally, on instruments from Stratagene; not required for instruments from Bio-Rad/MJ Research, Cepheid, Corbett, Eppendorf, and Roche
RNase-free water:	Ultrapure quality, PCR-grade

Quality Control

2x EpiTect MethyLight Master Mix	(See quality-control label inside kit lid for lot-specific values)
PCR sensitivity and reproducibility assay:	Sensitivity, reproducibility, and specificity in real-time PCR are tested in parallel 20 µl reactions containing 10-fold dilutions of nucleic acid template.
HotStarTaq *Plus* DNA Polymerase:	(included in the 2x EpiTect MethyLight PCR Master Mix) Efficiency and reproducibility in PCR are tested. Functional absence of exonucleases and endonucleases is tested.
Buffers and reagents	(included in the 2x EpiTect MethyLight PCR Master Mix).
EpiTect MethyLight PCR Buffer:	Conductivity and pH are tested.
RNase-free water:	Conductivity, pH, and RNase activities are tested.

Introduction

The determination of the relative prevalence of a particular pattern of methylated CpG dinucleotides in vertebrates is of particular interest in epigenetics research. MethyLight assays, probe-based real-time PCR for methylation analysis, are often used for sensitive quantification of the methylation pattern. Depending on the level used for sequence discrimination, MethyLight assays can be performed in different formats.

Quantitative MethyLight assays comprise PCR primers that do not overlap any CpG dinucleotides, the potential DNA methylation sites, and TaqMan® probes or other dual-labeled probes, which are located on a sequence containing the methylation sites of interest. The sequence discrimination therefore occurs at the level of probe hybridization — the methylation-specific probe can anneal to the methylated bisulfite converted DNA sequence whereas the unmethylation specific probe can bind to the unmethylated bisulfite converted DNA sequence. The primers used are located on bisulfite converted sequences without CpG sites.

In the case of EpiTect MethyLight Assays, the methylation specific TaqMan probe contains FAM™ as 5' reporter dye whereas the unmethylation specific TaqMan probe is linked to VIC®. Measuring the release of FAM and VIC during real-time PCR is then used to determine the methylation status (Figure 1), whereby the ratio of measured C_T values with both fluorescence dyes allows quantification of the methylation.

Semiquantitative MethyLight assays use methylation-specific primers in conjunction with a probe located in between. Thus, sequence discrimination occurs at the PCR amplification level. Two separate assays are required, one to determine the amount of methylated DNA, the other one to determine the amount of unmethylated DNA (Figure 2).

A mixture of the formats described is available for MethyLight assays. See reference 1.

EpiTect MethyLight PCR Kits are compatible with all MethyLight assay formats, using dual-labeled sequence-specific probes, providing flexible, rapid, and sensitive probe-based real-time PCR quantification of methylation status from CpG sites.

The kits are available in 2 formats:

- **EpiTect MethyLight PCR Kit**: This kit is supplied with a master mix containing ROX passive reference dye, and is optimized for use with real-time cyclers that require a high concentration of ROX dye for fluorescence normalization (e.g., instruments from Applied Biosystems, but not Applied Biosystems 7500 Real-Time PCR Systems).

- **EpiTect MethyLight PCR + ROX Vial Kit**: This kit is supplied with a master mix that is free of ROX dye, and also includes a separate solution of ROX dye which the user can add to reactions, depending on the real-time cycler used. The kit is intended for use with cyclers that require a lower concentration of ROX dye for fluorescence normalization (e.g., Applied Biosystems 7500 Real-Time PCR Systems), for use with cyclers that allow optional use of ROX dye (e.g., instruments from Stratagene), and for use with cyclers that do not require ROX dye. Running reactions without ROX dye allows greater flexibility when choosing reporter dyes for probes.

2x EpiTect MethyLight Master Mix

The components of the 2x EpiTect MethyLight Master Mix include HotStarTaq *Plus* DNA Polymerase, EpiTect MethyLight PCR Buffer, and ROX passive reference dye (see descriptions below). The 2x EpiTect MethyLight Master Mix (w/o ROX) contains HotStarTaq *Plus* DNA Polymerase and the EpiTect MethyLight PCR Buffer, but no ROX passive reference dye. The optimized master mixes ensure that the PCR products in a reaction with bisulfite converted DNA as starting material are amplified with high efficiency and sensitivity.

HotStarTaq *Plus* DNA Polymerase

HotStarTaq *Plus* DNA Polymerase is a modified form of QIAGEN *Taq* DNA Polymerase, and is provided in an inactive state and has no enzymatic activity at ambient temperature. This prevents the formation of misprimed products and primer–dimers during reaction setup and the first denaturation step. Competition for reactants by PCR artifacts is therefore avoided, enabling high PCR specificity and accurate quantification. The enzyme is activated at the start of a reaction by a 5-minute, 95°C incubation step. The hot start enables reactions to be set up rapidly and conveniently at room temperature.

2x EpiTect MethyLight Master Mix

The 2x EpiTect MethyLight Master Mix has been specifically developed for highly sensitive detection of methylated and unmethylated DNA using sequence-specific probes. In addition to various salts and additives, the buffer also contains a specially optimized combination of KCl and $(NH_4)_2SO_4$, which promotes a high ratio of specific to nonspecific primer and probe binding during the annealing step of each PCR cycle, allowing discriminative hybridization of the probes detecting unmethylated and/or methylated target sequences. The created stringent primer annealing conditions lead to increased PCR specificity when amplifying bisulfite converted DNA combined with reliable detection of the methylation degree. When using this buffer, primer annealing is only marginally influenced by $MgCl_2$ concentration so optimization by titration of Mg^{2+} is usually not required. The buffer also contains the synthetic Factor MP. This synthetic factor increases the local concentration of primers and probes at the DNA template and stabilizes specifically bound primers and probes, allowing efficient primer annealing and extension and supporting discriminative probe hybridization.

ROX passive reference dye

For certain real-time cyclers, the presence of ROX passive reference dye in real-time PCR compensates for non-PCR–related variations in fluorescence detection.

The use of ROX dye is necessary for instruments from Applied Biosystems and is optional for instruments from Stratagene. However, the presence of ROX dye in the master mix may limit the capabilities for combination of differentially labeled probes on some instruments. Therefore, we do not recommend using probes that have ROX or Texas Red® fluorophore as the reporter dye, since their performance in the presence of ROX passive reference dye is unpredictable. When performing reactions using probes labeled with Texas Red, ROX, or other equivalent fluorophore, use a real-time cycler that does not require ROX dye for fluorescence normalization.

The master mix supplied with the EpiTect MethyLight PCR Kit contains ROX dye at a concentration that is optimal for instruments from Applied Biosystems (models 7000, 7300, 7700, 7900HT, StepOne, and StepOnePlus, but not Applied Biosystems 7500 Real-Time PCR Systems).

For Applied Biosystems 7500 Real-Time PCR Systems and, optionally, instruments from Stratagene, ROX dye is required at a lower concentration. This is provided by the EpiTect MethyLight PCR Kit + ROX Vial, which requires the user to add the supplied ROX dye solution to reactions.

Instruments from all other suppliers, which do not require ROX dye for fluorescence normalization, should be used with the EpiTect MethyLight PCR Kit + ROX Vial, which provides master mix that does not contain ROX dye.

MethyLight Assays

EpiTect MethyLight PCR Kits can be used with all TaqMan probes, dual-labeled probes, and in all MethyLight assay formats.

Methylation-specific TaqMan probes

Methylation-specific TaqMan probes and dual-labeled probes are methylation-specific oligonucleotides with a fluorophore and a quencher moiety attached (Figure 1). The fluorophore is at the 5' end of the probe, and the quencher moiety is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5'→3' exonuclease activity of *Taq* DNA polymerase, separating the fluorophore and the quencher moiety. This results in detectable fluorescence that is proportional to the amount of accumulated PCR product.

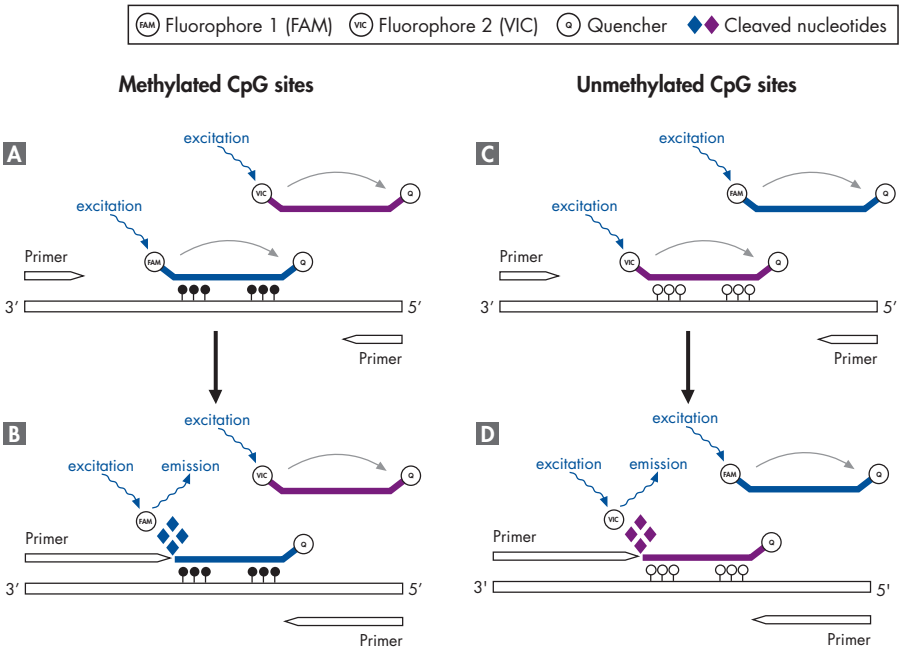

Figure 1. Principle of TaqMan probes used in conjunction with methylation-specific primers in quantitative, real-time PCR. In quantitative MethyLight Assays, TaqMan probes are located between primers which are located at methylation sites. In the case of methylated DNA, (**A**) primers specific to methylated sequences anneal to the DNA during the PCR annealing step. During the PCR extension step (**B**) *Taq* DNA Polymerase extends the primer. When the enzyme reaches the TaqMan probe annealed to the target sequence, its 5'→3' exonuclease activity degrades the probe, resulting in physical separation of the fluorophore from the quencher. In contrast, methylation-specific primers do not anneal to unmethylated, bisulfite converted DNA (**B**) and therefore, the TaqMan probe remains undegraded (**D**) and the proximity of the fluorophore with the quencher results in efficient quenching of fluorescence from the fluorophore.

TaqMan probes in conjunction with methylation-specific primers

In the second format of MethyLight Assays, TaqMan probes are used in between methylation-specific primer sites. Bisulfite converted DNA of unmethylated CpG sites require a different primer sequence to that of methylated CpG sites. Therefore, 2 different primer pairs are used, one specific for methylated and converted DNA, the other primer pair specific for unmethylated and converted DNA, which is the principle of MSP (methylation-specific PCR). If the methylation-specific primer binds to the DNA, it will be elongated during the extension phase and the 5'→3' exonuclease activity of *Taq* DNA Polymerase will lead to the degradation of the primer and the release of the fluorophore. As the fluorophore is now separated from the quencher moiety, a fluorescence is detectable (Figure 2).

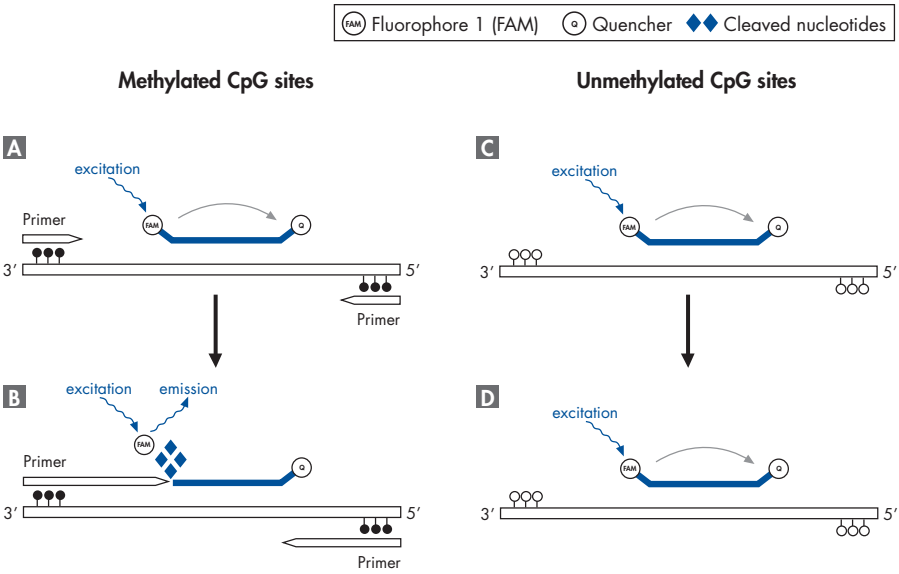

Figure 2. Principle of TaqMan probes used in conjunction with methylation-specific primers in semi-quantitative, real-time PCR. In semiquantitative MethyLight Assays, a TaqMan probe is located between primers which are located at the methylation sites. Primers specific for methylated sequences only anneal to methylated DNA (**A**) and not to unmethylated DNA (**C**). During the PCR extension step, the fluorophore is released and the resulting fluorescence is measured (**B**). In the case of unmethylated DNA, primer annealing and extension does not occur and fluorescence is not detected (**D**). Ideally a separate assay is applied, using primers specific for converted, unmethylated DNA, which determines the amount of unmethylated sequences. To quantify the methylation degree, a separate assay determining the DNA quantity is required (not shown in the figure).

For complete quantification, a separate assay is needed using primers specific to unmethylated and converted DNA, which measures the amount of unmethylated sequences.

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

- Primers and probes should be purchased from an established oligonucleotide manufacturer. Primers should be of standard quality, and probes should be HPLC purified. Lyophilized primers and probes should be dissolved in TE buffer to provide a stock solution of 100 µM; concentration should be checked by spectrophotometry (for details, see, page 29, "Dissolving primer and probes"). Primer and probe stock solutions should be stored in aliquots at –20°C. Probe stock solutions should be protected from exposure to light.
- If available, predesigned and validated EpiTect MethyLight Assays can be used. See our Ordering Information on page 31.
- Nuclease-free (RNase/DNase-free) consumables: Special care should be taken to avoid nuclease contamination of all reagents and consumables used to set up PCR for sensitive detection of DNA and cDNA targets.
- PCR tubes or plates (use thin-walled PCR tubes or plates recommended by the manufacturer of your real-time cycler).

Important Notes

Selecting kits and protocols

Select the correct EpiTect MethyLight PCR Kit and protocol to use with your real-time cycler.

See page 15 for recommended cyclers and the protocol for use with the EpiTect MethyLight PCR Kit and the ABI PRISM® 7000, Applied Biosystems 7300, ABI PRISM 7700, Applied Biosystems 7900 HT, Applied Biosystems StepOne, and Applied Biosystems StepOnePlus.

Refer to manufacturer's instructions for fluorescent dye detection capacity.

See page 19 for recommended cyclers and the protocol for use with the EpiTect MethyLight PCR + ROX Vial Kit and the Applied Biosystems 7500, iCycler iQ®, Mx3000P®, Mx3005P®, Mx4000®, Rotor-Gene™ 3000, SmartCycler® II, and other instruments.

Refer to manufacturer's instructions for fluorescent dye detection capacity.

No template control (NTC)

All methylation quantification experiments should include an NTC, containing all the components of the reaction except for the template. This enables detection of contamination.

To control the specificity of the MethyLight Assay to detect bisulfite converted DNA only, an initial experiment should be conducted using unconverted human genomic DNA, which should not be amplified. This DNA is part of the EpiTect PCR Control DNA Set (see Ordering Information on page 31).

Positive control

In some cases it may be necessary to include a positive control, containing a bisulfite converted DNA of known methylation status. Therefore, use of the EpiTect PCR Control DNA Set or a single EpiTect control DNA (e.g methylated or unmethylated human control DNA, bisulfite converted) is recommended. See page 31 for Ordering Information.

Protocol: Methylation-Specific Real-Time PCR Analysis Using TaqMan Probes (ABI 7000, 7300, 7700, 7900HT, StepOne, and StepOnePlus)

This protocol is optimized for use of the EpiTect MethyLight PCR Kit with TaqMan probes and other dual-labeled probes and real-time cyclers from Applied Biosystems **except Applied Biosystems 7500 Real-Time PCR Systems**. For further information, see "ROX passive reference dye", page 10.

Important points before starting

- Always start with the cycling conditions specified in this protocol.
- Use the primer and probe concentrations specified in this protocol.
- Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established MethyLight assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 10x primer–probe mix containing target-specific primers and probe. A 10x primer–probe mix for MethyLight PCR analysis consists of 4 µM forward primer, 4 µM reverse primer, and 2 µM probe in TE buffer.

Procedure

1. **Thaw the 2x EpiTect MethyLight Master Mix, primer and probe solutions, RNase-free water, and converted DNA. Mix the individual solutions, and place on ice.**
2. **Prepare a reaction mix according to Table 1 (page 16) or Table 2 (page 17).**

   For ease of use, we recommend preparing for each of your targets a 10x primer–probe mix containing primers and probe(s).

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided in the 2x EpiTect MethyLight Master Mix.
3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**

4. **Add template DNA (≤100 ng bisulfite converted DNA) to the individual PCR tubes or wells.**
5. **Program the real-time cycler according to Table 3 (page 17) or Table 4 (page 18).**

   Note: Check the real-time cycler's user manual for correct instrument setup (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for both reporter dyes used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

Table 1. Preparing reaction mix for methylation PCR analysis using TaqMan probes

Component	Volume per 50 µl reaction*	Final concentration
Reaction mix		
2x EpiTect MethyLight Master Mix	25 µl	1x
10x primer–probe mix†	5 µl	0.4 µM forward primer‡ 0.4 µM reverse primer‡ 0.2 µM probe (each, when using quantitative MethyLight assays)§
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable	≤100 ng/reaction
Total volume per reaction	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the Applied Biosystems 7900HT, use a reaction volume of 25 µl.

† A 10x primer–probe mix consists of 4 µM forward primer, 4 µM reverse primer, and 2 µM probe (each, when applying quantitative MethyLight assays) in TE buffer.

‡ A final primer concentration of 0.4 µM is optimal. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

§ A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 2. Preparing reaction mix for quantitative methylation PCR analysis using EpiTect MethyLight Assays

Component	Volume per 50 µl reaction*	Final concentration
Reaction mix		
2x EpiTect MethyLight Master Mix	25 µl	1x
MethyLight Primer Probe Mix, 10x	5 µl	1x
RNase-free water	Variable	–
Template DNA (added at step 4)	Variable	≤100 ng/reaction
Total volume per reaction	**50 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 25 µl, adjust the amount of master mix and all other reaction components accordingly. If using 384-well plates on the Applied Biosystems 7900HT, use a reaction volume of 25 µl.

Table 3. Cycling conditions for methylation PCR analysis using TaqMan probes

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/Extension	**60 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA

Table 4. Cycling conditions for quantitative methylation PCR analysis using EpiTect MethyLight Assays

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/Extension	**60 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. For each probe, select the analysis settings (i.e., baseline settings and threshold values). Note that optimal analysis settings are a prerequisite for accurate quantification data.

Protocol: Methylation-Specific Real-Time PCR Analysis Using TaqMan Probes (Applied Biosystems 7500 and Other Instruments)

This protocol is optimized for use of the EpiTect MethyLight PCR Kit + ROX Vial with TaqMan probes and other dual-labeled probes on Applied Biosystems 7500 Real-Time PCR Systems and on real-time cyclers which do not require ROX dye for fluorescence normalization (e.g., cyclers from Bio-Rad/MJ Research, Cepheid, Corbett, and Stratagene). For further information, see "ROX passive reference dye", page 10.

Important points before starting

- Always start with the cycling conditions specified in this protocol.
- Use the primer and probe concentrations specified in this protocol.
- Check whether your real-time cycler is compatible with the chosen combination of reporter dyes.
- If using an already established MethyLight assay, use the previously established primer and probe concentrations in combination with the cycling conditions specified in this protocol.
- **Optimal analysis settings are a prerequisite for accurate quantification data.** For data analysis, you should always readjust the analysis settings (i.e., baseline settings and threshold values) for analysis of every reporter dye channel in every run.
- When using the Applied Biosystems 7500 Fast Real-Time PCR System, select the Run Mode Standard 7500.

Things to do before starting

- For ease of use, we recommend preparing for each of your targets a 10x primer–probe mix containing target-specific primers and probe. A 10x primer–probe mix for MethyLight PCR analysis consists of 4 µM forward primer, 4 µM reverse primer, and 2 µM probe in TE buffer.

Procedure

1. **Thaw the 2x EpiTect MethyLight Master Mix (w/o ROX), 50x ROX Dye Solution, primer and probe solutions, RNase-free water, and converted DNA. Mix the individual solutions, and place on ice.**
2. **Prepare a reaction mix according to Table 5 (page 21) or Table 6 (page 22).**

   For ease of use, we recommend preparing for each of your targets a 10x primer–probe mix containing primers and probe(s).

   Note: We strongly recommend starting with the optimized Mg^{2+} concentration provided by the 2x EpiTect MethyLight Master Mix (w/o ROX). Due to the hot start, it is not necessary to keep samples on ice during reaction setup or while programming the real-time cycler.
3. **Mix the reaction mix thoroughly, and dispense appropriate volumes into PCR tubes or the wells of a PCR plate.**
4. **Add template DNA (≤100 ng bisulfite converted DNA) to the individual PCR tubes or wells.**
5. **Program the real-time cycler according to Table 7 (page 22) or Table 8 (page 23).**

   Note: Check the real-time cycler's user manual for correct instrument setup (e.g., setting up detection of multiple dyes from the same well). Be sure to activate the detector for both reporter dyes used. Depending on your instrument, it may also be necessary to perform a calibration procedure for each of the reporter dyes before they are used for the first time.

   When using the Applied Biosystems 7500 Fast Real-Time PCR System, select the Run Mode Standard 7500.

Table 5. Preparing reaction mix for methylation PCR analysis using TaqMan probes

Component	Volume per reaction 50 µl*	20 µl*†	Final concentration
Reaction mix			
2x EpiTect MethyLight Master Mix (w/o ROX)	25 µl	10 µl	1x
50x ROX Dye Solution‡	1 µl	0.4 µl	1x
10x primer–probe mix§	5 µl	2 µl	0.4 µM forward primer¶ 0.4 µM reverse primer¶ 0.2 µM probe (each, when using quantitative MethyLight assays)**
RNase-free water	Variable	Variable	–
Template DNA (added at step 4)	Variable	Variable	≤100 ng/reaction
Total volume per reaction	**50 µl***	**20 µl*†**	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly.

† Refers to the Applied Biosystems 7500 Fast Real-Time PCR System.

‡ For real-time cyclers that do not require ROX dye, add RNase-free water instead.

§ A 10x primer–probe mix consists of 4 µM forward primer, 4 µM reverse primer, and 2 µM probe (each, when applying quantitative MethyLight assays) in TE buffer.

¶ A final primer concentration of 0.4 µM is optimal. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

** A final probe concentration of 0.2 µM gives satisfactory results in most cases. Depending on the synthesis quality and purification method used, the optimal concentration may be between 0.1 µM and 0.4 µM.

Table 6. Preparing reaction mix for quantitative methylation PCR analysis using EpiTect MethyLight Assays

Component	Volume per 50 µl reaction*	Volume per 20 µl reaction*	Final concentration
Reaction mix			
2x EpiTect MethyLight Master Mix	25 µl	10 µl	1x
MethyLight Primer Probe Mix, 10x	5 µl	2 µl	1x
RNase-free water	Variable	Variable	–
Template DNA (added at step 4)	Variable	Variable	≤100 ng/reaction
Total volume per reaction	**50 µl***	**20 µl***	**–**

* If your real-time cycler requires a final reaction volume other than 50 µl or 20 µl, adjust the amount of master mix and all other reaction components accordingly.

Table 7. Cycling conditions for methylation PCR analysis using TaqMan probes

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/Extension	**60 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA

Table 8. Cycling conditions for quantitative methylation PCR analysis using EpiTect MethyLight Assays

Step	Time	Temperature	Additional comments
Initial PCR activation step	**5 min**	**95°C**	HotStarTaq *Plus* DNA Polymerase is activated by this heating step
2-step cycling:			**Important: Optimal performance is only assured using these cycling conditions**
Denaturation	**15 s**	**95°C**	
Annealing/Extension	**60 s**	**60°C**	Combined annealing/ extension step with fluorescence data collection
Number of cycles	40–45		The number of cycles depends on the amount of template DNA

6. **Place the PCR tubes or plate in the real-time cycler, and start the PCR cycling program.**
7. **Perform data analysis.**

   Before performing data analysis, specify the analysis settings. For each probe, select the analysis settings (i.e., baseline settings and threshold values). Note that optimal analysis settings are a prerequisite for accurate quantification data.

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 . The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

		Comments and suggestions
No signal, or one or more signals detected late in PCR		
a)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
b)	HotStarTaq *Plus* DNA Polymerase not activated	Ensure that the cycling program includes the HotStarTaq *Plus* DNA Polymerase activation step (5 min at 95°C) as described in the protocols.
c)	Pipetting error or missing reagent	Check the concentrations and storage conditions of the reagents, including primers, probes, and template nucleic acid. See Appendix A, page 28 for details on evaluating the concentration of primers and probes. Repeat the assay.
d)	Wrong or no detection step	Ensure that fluorescence detection takes place during the during the combined annealing/ extension step when using TaqMan probes.
e)	Primer or probe concentration not optimal	Use optimal primer concentrations. For details, see the individual protocols. In most cases, a probe concentration of 0.2 µM gives satisfactory results. Depending on the quality of your probe, results may be improved by adjusting probe concentration within the range of 0.1–0.4 µM. Check the concentrations of primers and probes by spectrophotometry (see Appendix A, page 28).

		Comments and suggestions
f)	Mg^{2+} concentration not optimal	The Mg^{2+} concentration in the 2x EpiTect MethyLight Master Mixes is already optimized. Increasing the final Mg^{2+} concentration by 0.5–1 mM may improve results.
g)	Problems with starting template	Ensure that the DNA is fully converted and therefore a suited template for methylation analysis using conversion-specific primers and probe(s). We recommend the EpiTect Bisulfite Kit. Use control DNAs as positive controls. Check the concentration, storage conditions, and quality of the starting nucleic acids. If necessary, make new serial dilutions of template nucleic acid from the stock solutions. Repeat the assay using the new dilutions. Ensure that all reagents, buffers, and solutions used for purification and dilution of template nucleic acids are free of nucleases.
h)	Insufficient amount of starting template	Increase the amount of template if possible. Ensure that sufficient copies of the target nucleic acids are present in your sample.
i)	Insufficient number of cycles	Increase the number of cycles.
j)	Probe design not optimal	If the amplification reaction was successful, there may be a problem with the probe. Review probe design.
k)	Wrong detection channel/filter chosen	Ensure that the correct detection channel is activated or the correct filter set is chosen for each reporter dye. Check whether the chosen combination of reporter dyes is compatible with the selected detection channels or filter sets.
l)	Fluorescence crosstalk	Check that the reporter dyes used in your assay are suitable for double probe analysis on your instrument. Run appropriate controls to estimate potential crosstalk effects.

		Comments and suggestions
m)	Wrong cycling conditions	Always start with the optimized cycling conditions specified in the protocols. Be sure that the cycling conditions include the initial step for activation of HotStarTaq *Plus* DNA Polymerase (95°C for 5 min), and the specified times for denaturation and annealing/extension.
n)	Analysis settings (e.g., threshold and baseline settings) not optimal	Check the analysis settings (threshold and baseline settings) for each reporter dye. Repeat analysis using optimal settings for each reporter dye.
o)	Use of (bisulfite untreated – unconverted) genomic DNA	Primers and probes in MethyLight assays are designed to prime bisulfite converted DNA only. Therefore, genomic DNA will not be amplified or detected. Check suitability of primers and probes with bisulfite converted control DNA.
No linearity in ratio of C_T value/crossing point to log of the template amount		
a)	Template amount too high	When signals are coming up at very early C_T values, adjust the analysis settings accordingly.
b)	Template amount too low	Increase template amount if possible. Note that detection of very low starting copy numbers may not be in the linear range of a standard curve.
Increased fluorescence or C_T value for "No Template" control		
a)	Contamination of reagents	Discard all the components of the multiplex assay (e.g., master mix, primers, and probes). Repeat the assay using new components.
b)	Minimal probe degradation, leading to sliding increase in fluorescence	Check the amplification plots, and adjust the threshold settings.
Varying fluorescence intensity		
a)	Contamination of real-time cycler	Decontaminate the real-time cycler according to the manufacturer's instructions.
b)	Real-time cycler no longer calibrated	Recalibrate the real-time cycler according to the manufacturer's instructions.

		Comments and suggestions
c)	Wavy curve at high template	In the analysis settings, reduce the number of cycles used for background calculation (if your real-time cycler allows you to do so) or reduce the amount of template.
d)	**ABI PRISM 7000**: Uneven curves or high standard deviations	Use the recommended reaction volume of 50 µl, and always use optical adhesive covers to seal plates.

Appendix A: Working with Bisulfite Converted DNA

Methylation occurs on cytosine residues in vertebrates, especially on CpG dinucleotides enriched in small regions of DNA. Methylation of CpG islands has been shown to be associated with gene inactivation and plays an important role in the development of cancer and cell aging.

The methylation status of a DNA can be determined by PCR only when using sodium bisulfite converted DNA. Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving methylated cytosines unchanged. For bisulfite conversion, we recommend the EpiTect Bisulfite Kit, which allows both conversion and cleanup.

Due to bisulfite conversion, the sequence will change, therefore DNA is no longer complementary and the primer for unmethylated DNA and methylated DNA differs in sequence. This can be used as a tool for checking methylation status of special CpGs.

Handling EpiTect MethyLight Assays and primers and probes

The EpiTect MethyLight Assays are developed to discriminate against the methylated and unmethylated allele in parallel, in one sample.

The ten-fold ready-to-use solution of the primer–probe mix in TE buffer can be directly used with the EpiTect MethyLight PCR Kit.

The EpiTect MethyLight Assays are developed and optimized for use with the EpiTect MethyLight PCR Kit. They are functionally validated with this kit.

Handling and storing primers and probes

Guidelines for handling and storing primers and probes are given below. For optimal results, we recommend only combining primers of comparable quality.

Storage buffer

Lyophilized primers and probes should be dissolved in a small volume of low-salt buffer to give a concentrated stock solution (e.g., 100 µM). We recommend using TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) for standard primers and probes labeled with most fluorescent dyes.

Storage

Primers should be stored in sterile, nuclease-free TE buffer in small aliquots at –20°C. Standard primers are stable under these conditions for at least 1 year. Fluorescently labeled probes are usually stable under these conditions for at least 6–9 months. Repeated freeze–thaw cycles should be avoided, since they may lead to degradation.

For easy and reproducible handling of primer–probe sets used in multiplex assays, we recommend preparing 20x primer–probe mixes, each containing the 2 primers and the probe for a particular target at the suggested concentrations.

Dissolving primers and probes

Before opening a tube containing lyophilized primer or probe, centrifuge the tube briefly to collect all material at the bottom of the tube. To dissolve the primer or the probe, add the required volume of sterile, nuclease-free TE buffer, mix, and leave for 20 minutes to allow the primer or probe to completely dissolve. Mix again and determine the concentration by spectrophotometry as described below.

We do not recommend dissolving primers and probes in water. They are less stable in water than in TE buffer and some may not dissolve easily in water.

Concentration

Spectrophotometric conversion for primers and probes:

1 A_{260} unit = 20–30 µg/ml

To check primer concentration, the molar extinction coefficient (ε_{260}) can be used:

$A_{260} = \varepsilon_{260}$ x molar concentration of primer or probe

If the ε_{260} value is not given on the data sheet supplied with the primers or probes, it can be calculated from the primer sequence using the following formula:

$\varepsilon_{260} = 0.89 \times [(A \times 15{,}480) + (C \times 7340) + (G \times 11{,}760) + (T \times 8850)]$

Example

Concentration of diluted primer: 1 µM = 1 x 10^{-6} M

Primer length: 24 nucleotides with 6 each of A, C, G, and T bases

Calculation of expected A_{260}: 0.89 x [(6 x 15,480) + (6 x 7340) + (6 x 11,760) + (6 x 8850)] x (1 x 10^{-6}) = 0.232

The measured A_{260} should be within ±30% of the theoretical value. If the measured A_{260} is very different to the theoretical value, we recommend recalculating the concentration of the primers or probes, or having the primers or probes resynthesized.

For probes, the fluorescent dye does not significantly affect the A_{260} value.

Primer and probe quality

The quality of 18–30mers can be checked on a 15% denaturing polyacrylamide gel;* a single band should be seen. Please contact QIAGEN Technical Services or your local distributor for a protocol or visit www.qiagen.com .

Probe quality

The quality of the fluorescent label and the purity of TaqMan probes can be determined by comparing fluorescence before and after DNase digestion. Incubate probes with or without 5 units DNase at 37°C for 1 hour. A significant difference in fluorescence following DNase treatment should be detectable.

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs) available from the product supplier.

Appendix B: Calculation of the Methylation Rate with EpiTect MethyLight Assays

Quantitative EpiTect MethyLight Assays enable the direct calculation of the methylation degree in a sample by taking the threshold cycles determined with each of both dyes:

Signal methylated:

$C_{T\,(CG)}$ (FAM) – represents the threshold cycle of the CG reporter (FAM channel)

Signal unmethylated:

$C_{T\,(TG)}$ (VIC) – represents the threshold cycle of the TG reporter (VIC channel)

Percentage of methylation: Cmeth = $100/[1+2^{(C_{T\,CG}-C_{T\,TG})}]\%$* (see reference 1)

Using the single probe-based MethyLight assay format, another calculation has to be performed. A commonly used method here is the determination of the PMR (percentage of fully methylated reference) (2).

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

For a complete list of references, visit the QIAGEN Reference Database online at www.qiagen.com/RefDB/search.asp or contact QIAGEN Technical Services or your local distributor.

Cited references

1. Eads, C.A. et al. (2000). MethyLight: A high-throughout assay to measure DNA Methylation. Nucleic Acid Res. **28**, e 32.
2. Cottrell et al. (2007). Discovery and Validation of 3 novel DNA methylation markers of prostate cancer prognosis. The Journal of Urology **177**, 1753.

Ordering Information

Product	Contents	Cat. no.
Related products		
EpiTect MethyLight Assays		
Hs_PITX2	10-fold primer TaqMan probe mix, for real-time MethyLight PCR	59930
Hs_PLAU	10-fold primer TaqMan probe mix, for real-time MethyLight PCR	59931
Hs_ONECUT2	10-fold primer TaqMan probe mix, for real-time MethyLight PCR	59932
Hs_ ERBB2	10-fold primer TaqMan probe mix, for real-time MethyLight PCR	59934
Hs_CDKN2A	10-fold primer TaqMan probe mix, for real-time MethyLight PCR	59933
Hs_TMEFF2	10-fold primer TaqMan probe mix, for real-time MethyLight PCR	59935
EpiTect Bisulfite Kits — for complete bisulfite conversion and cleanup of DNA for methylation analysis		
EpiTect Bisulfite Kit (48)	48 EpiTect Bisulfite Spin Columns, Reaction Mix, DNA Protect Buffer, Carrier RNA, Buffers	59104
EpiTect 96 Bisulfite Kit (2)	2 x EpiTect Bisulfite 96-well Plates, Reaction Mix, DNA Protect Buffer, Carrier RNA, Buffers	59110
EpiTect Control DNA — for evaluation of PCR primers used for methylation analysis		
EpiTect Control DNA, methylated (100)	Methylated and bisulfite converted human control DNA for 100 control PCRs	59655
EpiTect Control DNA, unmethylated (100)	Unmethylated and bisulfite converted human control DNA for 100 control PCRs	59665
EpiTect Control DNA (1000)	Unmethylated human control DNA for 1000 control PCRs	59568

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Product	Contents	Cat. no.
EpiTect PCR Control DNA Set (100)	Human control DNA set (containing both bisulfite converted methylated and unmethylated DNA and unconverted unmethylated DNA) for 100 control PCRs	59695
EpiTect Whole Bisulfitome Kit — for amplification of bisulfite converted DNA		
EpiTect Whole Bisulfitome Kit (25)	REPLI-g® Midi DNA Polymerase, EpiTect WBA Reaction Buffer, Nuclease-Free Water for 25 whole bisulfitome amplification reactions	59203
EpiTect Whole Bisulfitome (100)	REPLI-g Midi DNA Polymerase, EpiTect WBA Reaction Buffer, Nuclease-Free Water for 100 whole bisulfitome amplification reactions	59205
EpiTect MethyLight PCR Kit — for real-time quantification of methylation status		
EpiTect MethyLight PCR Kit (200)	Master Mix for methylation-specific real-time PCR analysis, 200 x 50 µl reactions	59436
EpiTect MethyLight PCR Kit (1000)	Master Mix for methylation-specific real-time PCR analysis, 1000 x 50 µl reactions	59438
EpiTect MethyLight PCR + ROX Vial Kit (200)	Master Mix without ROX for methylation-specific real-time PCR analysis, 200 x 50 µl reactions	59496
EpiTect MethyLight PCR + ROX Vial Kit (1000)	Master Mix without ROX for methylation-specific real-time PCR analysis, 1000 x 50 µl reactions	59498

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Notes

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