January 2020

EpiTect® Methyl II PCR Assay Handbook

For regional DNA methylation analysis using MethylScreen® technology

Sample to Insight

QIAGEN

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Storage

EpiTect Methyl II PCR Assays are shipped at ambient temperature. Upon receipt, store at –30 to –15°C in a constant-temperature freezer. If stored under these conditions, EpiTect Methyl II PCR Assays are stable for 6 months after receipt, unless otherwise indicated in the label.

Intended Use

EpiTect Methyl II PCR Assays are intended for molecular biology 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.

Safety Information

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

Quality Control

In accordance with QIAGEN's Quality Management System, each lot of EpiTect Methyl II PCR Assay is tested against predetermined specifications to ensure consistent product quality.

Introduction

The EpiTect Methyl II PCR System is an innovative technology enabling fast and accurate CpG island DNA methylation profiling of individual genes, as well as disease- and pathway-focused gene panels. The technology provides ready-to-use, predesigned primers to detect the methylation status of the promoter region (gene) of interest. Assays are ready-to-use primer pairs with high specificity and amplification efficiency. Predesigned primers offer genomewide coverage to detect the methylation status of the genes of interest. EpiTect Methyl II PCR Assays use the MethylScreen® technology provided under license from Orion Genomics, LLC (1).

Approximately 60–70% of all human gene promoters overlap with CpG islands — regions with an elevated GC content and a high frequency of CpG dinucleotides. Gene silencing by means of DNA methylation of specific genes promoters is a well-known feature of neoplastic cells and plays an important role in normal cell differentiation and development (2). DNA methylation occurs mainly at CpG dinucleotides and involves the enzymatic addition of a methyl group to the cytosine residue, without changing the primary DNA sequences.

Such modifications at regulatory regions (in particular gene promoters), correlate well with the transcriptional state of a gene: DNA methylation represses transcription while DNA unmethylation can lead to increased transcription levels. DNA methylation is an essential mechanism for normal cellular development, imprinting, x-chromosome inactivation, and maintaining tissue specificity. It can also contribute significantly to the progression of various human diseases.

The profiling of tumor suppressor genes and other key genes allows the correlation of CpG island methylation status with transcriptional status, biological phenotypes, or disease outcomes. Therefore, the results can provide insights into the molecular mechanisms and biological pathways and aid in the discovery and development of biomarkers.

Principle and procedure

The method employed by the EpiTect Methyl II PCR System is based on detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (3). These enzymes will digest unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA in each individual enzyme reaction is quantified by real-time PCR using primers that flank a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using a ΔC_T method. The reliability and simplicity of the procedure make this technology highly suited for semi-high-throughput DNA methylation profiling and biomarker development for various research fields, such as stem cell differentiation and development.

A protocol overview of the EpiTect Methyl II PCR Array is shown in Figure 1. Briefly, input genomic DNA is aliquoted into four equal portions and subjected to mock (no enzyme), methylation-sensitive (MSRE), methylation-dependent (MDRE), and double (MSRE and MDRE) restriction endonuclease digestion. After digestion, the enzyme reactions are mixed directly with qPCR master mix and are dispensed into a PCR Array plate containing prealiquoted primer mixes.

Real-time PCR is carried out using specified cycling conditions. Finally, the raw ΔC_T values are pasted into the data analysis spreadsheet, which automatically calculates the relative amount of methylated and unmethylated DNA fractions.

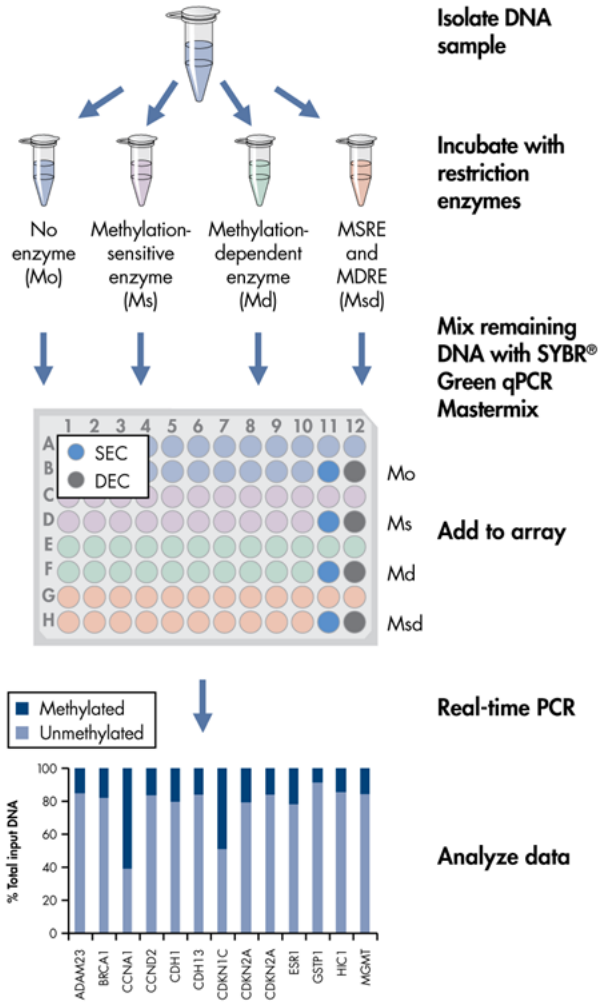


Figure 1. EpiTect Methyl II PCR procedure.

The product of the mock (no enzyme) digestion represents the total amount of input DNA for real-time PCR detection. In the methylation-sensitive digestion (M_s) reaction, the MSRE will digest unmethylated DNA, and then the remaining methylated DNA will be detected by real-time PCR. In the methylation-dependent digestion (M_d) reaction, the MDRE will digest methylated DNA, and then the remaining unmethylated DNA will be detected by real-time PCR. In the double digestion (M_{sd}) reaction, both enzymes are present, and all DNA molecules (both methylated and unmethylated) will be digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion.

EpiTect Methyl II PCR Assays provide gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. Unmethylated represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene. Methylated represents fraction of input genomic DNA containing 2 or more methylated CpG sites in the targeted region of a gene.

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

- DNeasy® Blood and Tissue Kit (cat. nos. 69504 or 69506) or the AllPrep® DNA/RNA Mini Kit (cat. no. 80204) for preparation of DNA
- EpiTect Methyl II DNA Restriction Kit (cat. no. 335452)

  Important: The EpiTect Methyl II DNA Restriction Kit contains all necessary components for cleavage of methylated and unmethylated DNA and is essential for a complete and successful experiment. The reagents included in the kit are sufficient for processing up to 12 µg of genomic DNA.
- Appropriate RT² SYBR® Green qPCR Mastermix (be sure to select the correct format for the PCR instrument, and size and quantity for the number of samples):
  - RT² SYBR® Green ROX™ qPCR Mastermix (cat. nos. 330520, 330522, 330523, 330521, 330529) for all Applied Biosystems® and Stratagene® instruments, and Eppendorf® Mastercycler® ep realplex instruments with a ROX filter set
  - RT² SYBR® Green Fluor qPCR Mastermix (cat. nos. 330510, 330510, 330513, 330511, 330519) for Bio-Rad® iCycler®, MyiQ™, MyiQ2™, and iQ™5 instruments
  - RT² SYBR® Green qPCR Mastermix (cat. nos. 330500, 330502, 330503, 330501, 330509) for instruments that do not require a reference dye, e.g., Bio-Rad models CFX96™, CFX384™, Bio-Rad/MJ Research Opticon 2, and Bio-Rad/MJ Research Chromo4™, Roche® LightCycler® 480 (96- and 384-well)

- Real-time PCR instrument
- Calibrated single- and multi-channel pipettes
- RNase-/DNase-free pipette tips and tubes
- RNase-/DNase-free 100 µl regular PCR tubes (8- or 12-tube strings)
- Molecular-biology–grade RNase- and DNase-free water

Important Notes

Controls for monitoring enzyme digestion efficiency

A successful EpiTect Methyl II PCR experiment relies on efficient DNA digestion by methylation-sensitive and methylation-dependent restriction enzymes. We recommend using the EpiTect Methyl II PCR Assay for EP_SEC (cat. no. EPHS115450-1A) and EP_DEC (cat. no. EPHS115451-1A) to test the cutting efficiency of the restriction enzymes and to ensure reliable and reproducible results. The C_T values from the real-time PCR are pasted into the Microsoft® Excel® data analysis spreadsheet, and a "Pass" or "Fail" result is returned for the SEC (methylation-sensitive enzyme control) and DEC (methylation-dependent enzyme control) assays.

DNA contamination

For reliable results, it is very important to prevent contamination of the EpiTect Methyl II PCR Assay reactions with foreign DNA. Even very small amounts of foreign DNA can artificially inflate SYBR® Green signals, yielding false positive results. The most common source of contamination in the PCR reagents comes from the products of previous PCR experiments in your working area. To minimize contamination, follow the recommendations below:

- Wear gloves throughout the entire procedure.
- Use only fresh PCR-grade reagents and labware.
- Physically separate the workspace for PCR setup and post-PCR work.
- Before setting up an experiment, decontaminate the PCR workspace and labware (pipette barrels, tube racks, etc.) with 10% bleach and UV light. Preferentially set up reactions in a PCR workstation.
- Do not remove the protective film from the PCR array until immediately before use.

- Close all tubes containing PCR products as soon as possible after use.
- Treat any labware (tips or tubes) containing PCR products or other DNA with 10% bleach before discarding.

Genomic DNA preparation

High-quality DNA is a prerequisite for a successful EpiTect Methyl II PCR Assay reaction. Therefore, sample handling and genomic DNA isolation procedures are crucial to the success of the experiment. Residual traces of proteins, salts, or other contaminants will either degrade the DNA or decrease the restriction enzyme activities necessary for optimal DNA digestion.

We recommend the DNeasy Blood and Tissue Kit or the AllPrep DNA/RNA Mini Kit for preparation of genomic DNA samples. Regardless of the DNA isolation kit used, ensure that samples have been treated for the removal of RNA, because RNA contamination will cause inaccuracies in DNA concentration measurements and may affect restriction digestion efficiency. Do not omit the recommended RNase-treatment step to remove RNA. If genomic DNA samples are harvested from biological samples where purification kits are not available, contact QIAGEN Technical Services for suggestions.

For best results, resuspend or dilute all DNA samples in DNase-free water, or alternatively, in DNase-free 10 mM Tris buffer pH 8.0 without EDTA.

Measurement of DNA concentration and purity by UV spectrophotometry

Prepare dilutions of genomic DNA samples and measure absorbance in DNase-free 10 mM Tris buffer, pH 8.0. The spectral properties of nucleic acids are highly dependent on pH. The recommended ratios and values for DNA are as follows:

- A_{260}/A_{230}: >1.7
- A_{260}/A_{280}: >1.8
- A_{260} concentration: >4 µg/ml

DNA concentrations for restriction digestion and PCR assay

Using the recommended amount of DNA optimizes the sensitivity of detecting methylated DNA. More input DNA may be used if analyzing methylated DNA isolated from samples of heterogeneous cell types (e.g., tumor samples) where heavy nontumor cell contamination (e.g., blood, stromal cells, etc.) is expected. However, maintain the specific enzyme-to-DNA ratios outlined below for each assay, and purchase additional qPCR plates to ensure assay consistency.

Using the EpiTect Methyl II DNA Restriction Kit

- **Important**: Do not vortex enzymes.

  Methylation-dependent enzyme B is very sensitive to vortexing. Extensive vortexing may cause a loss of enzyme activity. Mix enzymes by pipetting gently up and down.
- Store enzymes at –20°C. When in use, enzymes should be kept on ice.

Protocol: EpiTect Methyl II PCR Assay for 5 Genes Using 1 DNA Sample

Be sure to read “Important Notes”, page 11, before starting the protocol.

Procedure

Restriction digestion

1. Perform the restriction digestions using the EpiTect Methyl II DNA Restriction Kit.
2. Prepare a reaction mix without enzymes as indicated in Table 1.

   We recommend using 250 ng genomic DNA. The 5x Restriction Digestion Buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer, continue mixing the buffer until precipitates dissolve.

Table 1. Reaction mix without enzymes

Component	Volume
Genomic DNA (250 ng)	Variable
5x Restriction Digestion Buffer	26 µl
RNase-/DNase-free water	Variable
Final volume	**120 µl**

3. Add RNase-/DNase-free water to make the final volume 120 µl. Vortex to thoroughly mix the components and centrifuge briefly in a microcentrifuge.

4. Set up 4 digestion reactions (M_o, M_s, M_d, and M_{sd}) according to Table 2.

   Important: All 4 tubes must contain equal amounts of genomic DNA.

Table 2. Restriction digestion

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	28 µl	28 µl	28 µl	28 µl
Methylation-sensitive enzyme A	–	1 µl	–	1 µl
Methylation-dependent enzyme B	–	–	1 µl	1 µl
RNase-/DNase-free water	2 µl	1 µl	1 µl	–
Final volume	**30 µl**	**30 µl**	**30 µl**	**30 µl**

5. Pipet up and down to gently, but thoroughly mix the components. Centrifuge the tubes briefly in a microcentrifuge.

   Important: Do not vortex!

6. Incubate all 4 tubes at 37°C for 6 h in a heating block or thermal cycler. The reaction can also be performed overnight.
7. After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 min.
8. The reactions are now ready for use or storage at –30 to –15°C. Mix the samples thoroughly by vortexing before use. Centrifuge the samples briefly and proceed to step 1 of "Setting up the PCR".

Setting up the PCR

1. Prepare individual reactions for each of the 4 digestions (M_o, M_s, M_d, and M_{sd}) in a 1.5 ml tube according to Table 3 (96-well plate) or Table 4 (384 well plate). Repeat for each gene (up to 5).

Table 3. PCR setup (96-well plate)

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	12.5 µl	12.5 µl	12.5 µl	12.5 µl
PCR primer mix	1.0 µl	1.0 µl	1.0 µl	1.0 µl
M_o digest	5 µl	–	–	–
M_s digest	–	5 µl	–	–
M_d digest	–	–	5 µl	–
M_{sd} digest	–	–	–	5 µl
RNase-/DNase-free water	6.5 µl	6.5 µl	6.5 µl	6.5 µl
Final volume	**25 µl**	**25 µl**	**25 µl**	**25 µl**

Table 4. PCR setup (384-well plate)

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	5 µl	5 µl	5 µl	5 µl
PCR primer mix	0.4 µl	0.4 µl	0.4 µl	0.4 µl
M_o digest	2 µl	–	–	–
M_s digest	–	2 µl	–	–
M_d digest	–	–	2 µl	–
M_{sd} digest	–	–	–	2 µl
RNase-/DNase-free water	2.6 µl	2.6 µl	2.6 µl	2.6 µl
Final volume	**10 µl**	**10 µl**	**10 µl**	**10 µl**

2. Mix tubes well by vortexing, and briefly centrifuge the contents to the bottom of the tube.
3. Add 25 µl of each reaction to the EpiTect Methyl II PCR Assay 96-well plate, as shown in Table 5. For 384-well plates, use 10 µl.

   Note: If more than one sample is run, plates can be further loaded in columns 6–12. Ensure that same row pattern is followed for loading (M_o in row A, M_s in row C, M_d in row E, and M_{sd} in row G).

Table 5. Assay setup (96-well)

Reaction	Gene 1	Gene 2	Gene 3	Gene 4	Gene 5
M_o digest	A1	A2	A3	A4	A5
M_s digest	C1	C2	C3	C4	C5
M_d digest	E1	E2	E3	E4	E5
M_{sd} digest	G1	G2	G3	G4	G5

4. Seal or cap the wells of the plate. Centrifuge the plate for 1 min at 2000 rpm to remove any air bubbles.

Running the PCR

1. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 6.

   Note: It is critical that the cycling conditions are followed exactly.

Table 6. PCR cycling protocol

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

* Hot start to activate DNA polymerase.

† Detect and record SYBR® Green fluorescence from each well during the annealing step of each cycle.

2. After the run has finished, analyze the data as described in "Data Analysis", page 22.

Protocol: EpiTect Methyl II PCR Assay for 2 Genes Using 1 DNA Sample

Be sure to read “Important Notes”, page 11, before starting the protocol.

Procedure

Restriction digestion

1. Perform the restriction digestions using the EpiTect Methyl II DNA Restriction.
2. Prepare a reaction mix without enzymes as indicated in Table 7.

   We recommend using 125 ng genomic DNA. The 5x Restriction Digestion Buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer, continue mixing the buffer until precipitates dissolve.

Table 7. Reaction mix without enzymes

Component	Volume
Genomic DNA (125 ng)	Variable
5x Restriction Digestion Buffer	13 µl
RNase-/DNase-free water	Variable
Final volume	**60 µl**

3. Add RNase-/DNase-free water to make the final volume 60 µl. Vortex to thoroughly mix the components and centrifuge briefly in a microcentrifuge.
4. Set up 4 digestion reactions (M_o, M_s, M_d, and M_{sd}) according to Table 8.

   Important: All 4 tubes must contain equal amounts of genomic DNA.

Table 8. Restriction digestion

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	14 µl	14 µl	14 µl	14 µl
Methylation-sensitive enzyme A	–	0.5 µl	–	0.5 µl
Methylation-dependent enzyme B	–	–	0.5 µl	0.5 µl
RNase-/DNase-free water	1 µl	0.5 µl	0.5 µl	–
Final volume	**15 µl**	**15 µl**	**15 µl**	**15 µl**

5. Pipet up and down to gently, but thoroughly mix the components. Centrifuge the tubes briefly in a microcentrifuge.

   Important: Do not vortex!

6. Incubate all 4 tubes at 37°C for 6 h in a heating block or thermal cycler. The reaction can also be performed overnight.
7. After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 min.
8. The reactions are now ready for use or storage at –30 to –15°C. Mix samples thoroughly by vortexing before use. Centrifuge samples briefly and proceed to step 1 of "Setting up the PCR".

Setting up the PCR

1. Prepare individual reactions for each of the 4 digestions (M_o, M_s, M_d, and M_{sd}) in a 1.5 ml tube according to Table 9 (96-well plate) or Table 10 (384-well plate). Repeat for each gene (up to 2).

Table 9. PCR setup (96-well plate)

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	12.5 µl	12.5 µl	12.5 µl	12.5 µl
PCR primer mix	1.0 µl	1.0 µl	1.0 µl	1.0 µl
M_o digest	5 µl	–	–	–
M_s digest	–	5 µl	–	–
M_d digest	–	–	5 µl	–
M_{sd} digest	–	–	–	5 µl
RNase-/DNase-free water	6.5 µl	6.5 µl	6.5 µl	6.5 µl
Final volume	**25 µl**	**25 µl**	**25 µl**	**25 µl**

Table 10. PCR setup (384-well plate)

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	5 µl	5 µl	5 µl	5 µl
PCR primer mix	0.4 µl	0.4 µl	0.4 µl	0.4 µl
M_o digest	2 µl	–	–	–
M_s digest	–	2 µl	–	–
M_d digest	–	–	2 µl	–
M_{sd} digest	–	–	–	2 µl
RNase-/DNase-free water	2.6 µl	2.6 µl	2.6 µl	2.6 µl
Final volume	**10 µl**	**10 µl**	**10 µl**	**10 µl**

2. Mix tubes well by vortexing, and briefly centrifuge the contents to the bottom of the tube.
3. Add 25 µl of each reaction to the EpiTect Methyl II PCR Assay 96-well plate, as shown in Table 11. For 384-well plates, use 10 µl.

   Note: If more than one sample is run, plates can be further loaded in columns 3–12. Ensure that same row pattern is followed for loading (M_o in row A, M_s in row C, M_d in row E, and M_{sd} in row G).

Table 11. Assay setup (96-well)

Reaction	Gene 1	Gene 2
M_o digest	A1	A2
M_s digest	C1	C2
M_d digest	E1	E2
M_{sd} digest	G1	G2

4. Seal or cap the wells of the plate. Centrifuge the plate for 1 min at 2000 rpm to remove any air bubbles.

Running the PCR

1. Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 12.

   Note: It is critical that the cycling conditions are followed exactly.

Table 12. PCR cycling protocol

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

* Hot-start to activate DNA polymerase.

† Detect and record SYBR® Green fluorescence from each well during the annealing step of each cycle.

2. After the run has finished, analyze the data as described in "Data Analysis", page 22.

Data analysis

Obtaining raw threshold cycle (C_T) values

After the cycling program has completed, obtain the C_T values according to the instructions provided by the manufacturer of the real-time PCR instrument. We recommend manually setting the baseline and threshold values as follows.

Note: When comparing multiple plates, make sure that the settings for all plates are identical.

Baseline: Using the Linear View of the amplification plots, set the instrument to use the readings from cycle number 2 through the cycle just before the earliest visible amplification, usually between cycle 10 and 15.

Threshold value: Using the Log View of the amplification plots, place the threshold above the background signal but within the lower third of the linear portion of the amplification curves.

Exporting C_T values

Export and/or copy-paste the C_T values from the instrument software to a blank Microsoft Excel spreadsheet according the manufacturer's instructions for the real-time PCR instrument.

Microsoft Excel based data analysis template

First, download the relevant EpiTect Methyl II PCR Array Microsoft Excel–based data analysis template, which is available at **geneglobe.qiagen.com/product-groups/epitect-methyl-ii-pcr-arrays**.

Then, paste in the C_T value data and analyze the automatically generated results by following the directions in the "Instructions" worksheet of the Excel file.

Data quality control

Mock digest (M_o) C_T values

The C_T values of the mock digests for all genes should be within the range of 18 to 27 cycles, if the recommended amounts of genomic DNA were used.

Single enzyme digest (M_s and M_d) C_T values

The C_T values of the M_s and M_d digests should be between the values of the mock and double digests, depending on the methylation status of the DNA samples.

Double digest (Msd) C_T values

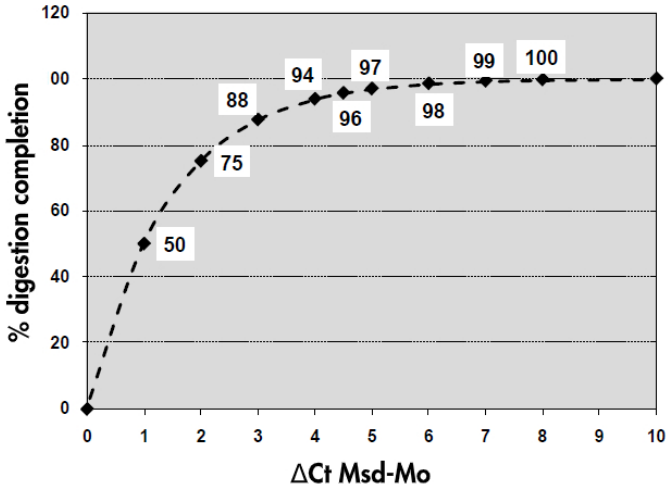
The C_T values of the double digests should be higher than the C_T values of the mock digest.

Enzyme digestion efficiency

The difference in C_T values between the double digest and mock digest samples represents the analytical window (W) of the assay and should be greater than 3 (ΔC_T [M_{sd} – M_o] > 3). This means that more than 87.5% of all DNA molecules in the samples were digested, and that the assay results are reliable and meaningful. See also the "Data QC Report" worksheet in the Microsoft Excel data analysis template. For every gene, the analytical window (W) values should be >3 and the F_R values should be <12.5%.

When using the EpiTect Methyl II PCR Assay for EP_SEC (cat. no. EPHS115450-1A) and EP_DEC (cat. no. EPHS115451-1A) to test the cutting efficiencies of the restriction enzymes, copy and paste the C_T values for these controls into the Microsoft Excel data analysis template. For the SEC assay, the difference in C_T values between the methylation-sensitive and mock digests should be equal to or greater than 4 (ΔC_T (M_s – M_o) ≥ 4) to pass the quality control.

Likewise for the DEC control assay, the difference in C_T values between the methylation-dependent and mock digests should be equal to or greater than 4 (ΔC_T (M_d – M_o) ≥ 4) to pass the quality control. "Pass" means that more than 93.6% of control DNA molecules spiked in buffer (5x Restriction Digestion Buffer) were digested, showing that the restriction enzymes were active and digested the DNA efficiently. See the "Result" worksheet in the Microsoft Excel data analysis template. Both the SEC and DEC assays should show "Pass" in the analysis.



Data interpretation

EpiTect Methyl II PCR Assays provide gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. "UM" represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene. "M" represents the fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene. The number of CpG sites methylated in a targeted region can vary within the fraction of methylated input DNA.

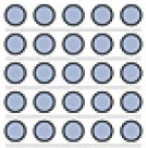
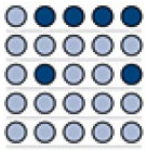
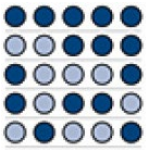
Example #	Input genomic DNA	Final results	
		Unmethylated (UM)	Methylated (M)
1		100%	0%
2		60%	40%
3		0%	100%
4		0%	100%

Figure 2. Pictorial explanations of results.

In Figure 2, each horizontal bar represents the targeted region of a gene from one genome. Biological samples usually contain many genomes derived from many cell types. For simplicity, five such genomes are depicted here. Light and dark circles represent unmethylated and methylated CpG sites, respectively.

The "Results" worksheet displays the relative percentage of methylated (M) and unmethylated (UM) DNA in each target genomic DNA sequence. Significance of the level of methylated DNA (% of total input DNA) must be defined by the researcher. Methylated DNA may have

biological significance if such methylation status is associated with a specific tumor, tissue, or other phenotype. Ideally, to determine if this methylation status is sufficient to repress transcription, measuring the corresponding expression levels and comparing those with the expression levels in the appropriate controls should be considered. Alternatively, results can be compared between a control and experimental DNA samples. Such parallel analysis will allow researchers to see if the methylation status of an experimental sample is substantially different from a matched control sample (i.e., tumor sample vs. normal control or treated sample vs. untreated).

ΔC_T data analysis

Due to the inversely proportional relationship between threshold cycle and the amount of input DNA, and due to the doubling of PCR product with every cycle in the exponential phase of the reaction, the initial DNA amount in each digest before PCR is expressed as:

$$C_{M_o} = 2^{-C_T(M_o)};\ C_{M_s} = 2^{-C_T(M_s)};\ C_{M_d} = 2^{-C_T(M_d)};\ C_{M_{sd}} = 2^{-C_T(M_{sd})} \quad (1)$$

The fraction of DNA in each digest is calculated by normalizing the DNA amount to the amount of digestible DNA. The amount of digestible DNA is equal to the total amount of DNA (determined from the mock digest) minus the amount of DNA resistant to DNA digestion (determined from the double digest).

Unmethylated (UM) DNA fraction:

$$F_{UM} = \frac{C_{M_d}}{C_{M_o} - C_{M_{sd}}} = \frac{2^{-C_T(M_d)}}{2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}} \quad (2)$$

Hypermethylated (HM) DNA fraction:

$$F_{HM} = \frac{C_{M_s}}{C_{M_o} - C_{M_{sd}}} = \frac{2^{-C_T(M_s)}}{2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}}$$

Intermediately methylated (IM) DNA fraction:

$$F_{IM} = 1 - F_{HM} - F_{UM}$$

Methylated (M) DNA fraction:

$$F_M = F_{HM} + F_{IM} \quad (3)$$

DNA copies resistant (R) to enzyme digestion:

$$F_R = \frac{C_{M_{sd}}}{C_{M_o}} \quad (4)$$

Example:

Symbol	**M_o**	**M_s**	**M_d**	**M_{sd}**	**R**	**UM**	**M**
CCNA1	23.16	27.11	24.89	36.51	0.0095%	30.15%	69.85%

F_{UM} = 2 ^ – (C_T M_d) / (2 ^ (– C_T M_o) – 2^ (– C_T M_{sd})) = 0.3015 or 30.15%

F_{HM} = 2 ^ – (C_T M_s) / (2 ^ (– C_T M_o) – 2^ (– C_T M_{sd})) = 0.0647 or 6.47%

F_{IM} = 1 – F_{HM} – F_{UM} = 1 – 0.0647 – 0.3015 = 0.6338 or 63.38%

F_M = F_{HM} + F_{IM} = 0.0647 + 0.6338 = 0.6985 or 69.85%

F_R = 2 ^ – (C_T M_{sd} – C_T M_o) = 2 ^ – (36.51 – 23.16) = 0.0095%

Methylation-sensitive or methylation-dependent digest C_T values within one cycle of the mock digest cannot be reliably used to calculate the percentage of either respective methylated DNA fraction. Differences in threshold cycles less than one (1) are within the standard error associated with real-time PCR instruments and experimental procedures. In these situations, the digest with the greatest difference in C_T value from the mock digest is used to calculate its DNA fraction, whether unmethylated or methylated. The opposite fraction (methylated or unmethylated, respectively) is instead calculated as one minus the determined fraction.

If ΔC_T (M_s – M_o) < 1.0 and ΔC_T (M_d – M_o) > 1.0, use following formula to calculate the fraction of methylated DNA:

$$F_M = 1 - F_{UM} = 1 - [C_{M_d} / (C_{M_o} - C_{M_{sd}})] \quad (5)$$

Example:

Symbol	**M_o**	**M_s**	**M_d**	**M_{sd}**	**R**	**M**	**UM**
CDH13	19.84	20.60	28.38	30.36	0.068%	99.73%	0.27%

F_{UM} = 2 ^ – (C_T M_d) / [2 ^ (– C_T M_o) – 2^ (– C_T M_{sd})] = 0.0027 or 0.27%

$F_M = 1 - F_{UM} = 1 - 0.0027 = 0.9973$ or 99.73%

F_R = 2 ^ – (C_T M_{sd} – C_T M_o) = 2 ^ – (36.36 – 19.84) = 0.068%

If ΔC_T (M_d – M_o) < 1.0 and ΔC_T (M_s – M_o) > 1.0, use following formula to calculate the fraction of unmethylated DNA:

$$F_{UM} = 1 - F_M = 1 - (C_{M_s} / (C_{M_o} - C_{M_{sd}})) \quad (6)$$

Example:

Symbol	M_o	M_s	M_d	M_{sd}	R	M	UM
BRCA1	22.96	32.73	23.34	40.00	0.0007%	0.11%	99.89%

$F_M = F_{HM} = 2 \text{ ^ } – (C_T M_s) / (2 \text{ ^ } (– C_T M_o) – 2\text{^ } (– C_T M_{sd})) = 0.00114$ or 0.11%

$F_{UM} = 1 – F_{HM} = 1 – 0.00114 = 0.99888$ or 99.89%

$F_R = 2 \text{ ^ } – (C_T M_{sd} – C_T M_o) = 2 \text{ ^ } – (40.00 – 22.96) = 0.0007\%$

If both ΔC_T (M_s – M_o) and ΔC_T (M_d – M_o) are less than 1.0, then the fraction of both methylated and unmethylated DNA is assigned as 50%.

Example:

Symbol	M_o	M_s	M_d	M_{sd}	R	M	UM
SFN	24.03	24.03	24.59	40.00	0.0016%	50.0%	50.0%

$F_M = F_{HM} = F_{UM} = 50.0\%$

$F_R = 2 \text{ ^ } – (C_T M_{sd} – C_T M_o) = 2 \text{ ^ } – (40.00 – 24.03) = 0.0016\%$

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 the information and/or protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
Incomplete restriction enzyme digestion: C_T (M_{sd}) – C_T (M_o) < 3 or SEC or DEC "Fail" in the data analysis worksheet	
a) Poor-quality DNA	Repeat the experiment with new DNA samples.
b) Low restriction enzyme activity	Check that the EpiTect Methyl II DNA Restriction Kit has not expired. Be sure to use the correct amount of both enzymes recommended in the protocol for the amount of DNA used. Be sure to mix the enzyme by gently pipetting up and down.
c) RNA contamination in the DNA samples	RNA contamination inhibits restriction enzyme DNA digestion and causes an overestimation of DNA concentration. Be sure to include any RNase treatment steps recommended in the procedure of the chosen DNA preparation kit.
d) Other contaminants in the DNA samples	DNA prepared from difficult organ tissues may contain protein and/or polysaccharide contaminants that significantly inhibit restriction enzyme activity. Organic reagents (such as chloroform, phenol, and isopropanol) used in some DNA kits and protocols may not be completely removed. Be sure to use the recommended DNA isolation kits and protocols and avoid using organic solvent-based methods and protocols for DNA preparation.
e) Too much DNA used in the digestion	Carefully measure the DNA concentration and only use the amount of DNA recommended by the selected PCR setup protocol.
f) Incorrect incubation conditions	Incubate for at least 4 hours at 37°C and use the size of tubes recommended in the protocol. Use an overnight incubation if a shorter time was used previously and resulted in incomplete digestion.

	Comments and suggestions
High mock digestion (M_o) C_T values from most/all genes	
a) Insufficient DNA used in the digestion	Be sure to use at least the amount of DNA recommended in the protocol. Use the recommended methods and instruments to determine DNA concentrations. Be sure to include any RNase treatment steps recommended in the procedure of the chosen DNA isolation kit.
b) Degraded DNA	DNA samples may be contaminated by microbes due to improper storage of DNA samples, e.g., at 4°C. Always store DNA samples at –30 to –15°C (up to 2 years) or –80 °C (indefinitely).
c) PCR array or master mix incorrectly stored	Storing PCR array or master mix at inappropriate temperature for extended periods reduces their activity and PCR amplification efficiency.
d) Incorrect real-time PCR cycling program used	Be sure to use the correct cycling program, including 10 minutes at 95°C to fully activate the hot start enzyme in the RT2 SYBR Green qPCR Mastermix.
All 4 digests (M_o, M_s, M_d, M_{sd}) CT values for an individual gene are ≥32	
a) DNA sample may contain a different sequence relative to the most recent NCBI genome build	This may be due to unreported chromosomal abnormalities (insertion or deletions) or single nucleotide polymorphisms (SNPs) that affect the EpiTect Methyl II PCR Assays. Verification may require sequencing of the relevant genomic region in the original DNA sample.
b) Homozygous deletions	If the CT values from all 4 digests for an individual gene, but not the majority of genes, are ≥32, genomic homozygous deletion most likely exists at this locus in the genomic DNA of the original sample.

References

1. Holemon H., et al. (2007) MethylScreen: DNA methylation density monitoring using quantitative PCR. Biotechniques **43**, 683–693.
2. Esteller, M. (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum. Mol. Genet. **16 Spec No 1**, R50–R59.
3. Ordway, J.M., et al. (2006) Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets. Carcinogenesis **27**, 2409–2423.

Ordering Information

Product	Contents	Cat. no.
EpiTect Methyl II PCR Assay (200)	Laboratory-tested forward and reverse primers for 200 x 25 µl reactions; 25 µl per primer; total volume: 200 µl	335002
Related products		
EpiTect Methyl II Signature PCR Array (22)	For methylation analysis of 22 genes in a 96-well or 384-well plate format2, 12, or 24 x 96; 4 x 384	335212
EpiTect Methyl II Complete PCR Array (94)	For methylation analysis of 94 genes in a 96-well or 384-well plate format2 x set of 4 of 96; 2, 12, or 24 x 384	335222
EpiTect Methyl II Custom PCR Array	For methylation analysis of customer- selected genes in a 96-well or 384-well plate format	335112
EpiTect Methyl II DNA Restriction Kit (12)	Reagents for the cleavage of methylated and unmethylated DNA for processing up to 12 DNA samples; 5x Restriction Digestion Buffer, Methylation-Sensitive Enzyme A, Methylation-Dependent Enzyme B	335452
DNeasy Blood and Tissue Kit (50)*	50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
AllPrep DNA/RNA Mini Kit (50)	For 50 minipreps: AllPrep DNA Spin Columns, RNeasy® Mini Spin Columns, Collection Tubes, RNase-Free Water and Buffers	80204

* Larger kit sizes available; please inquire.

Product	Contents	Cat. no.
RT² SYBR® Green qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that do not require a reference dye, including Bio-Rad models CFX96, CFX384, Bio-Rad/MJ Research Chromo4, Bio-Rad/MJ Research Opticon 2; Roche LightCycler 480 (96-well and 384-well); and all other cyclers	330500
RT2 SYBR® Green ROX qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with the following real-time cyclers: Applied Biosystems models 5700, 7000, 7300, 7500 [Standard and Fast], 7700, 7900HT 96-well block [Standard and Fast] and 384-well block, StepOnePlus; Eppendorf Mastercycler ep realplex models 2, 2S, 4, 4S; Stratagene models Mx3000P, Mx3005P, Mx4000	330520
RT² SYBR® Green Fluor qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with the following real-time cyclers: Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2	330510
RT² PCR Array Loading Reservoir	5 ml capacity reservoir for convenient sample loading on PCR arrays	338162

* Larger kit sizes available; please inquire.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
01/2020	Replaced sabiosciences.com link with qiagen.com link. Removed statement suggesting that the user can use hierarchical clustering to generate a graphic representation of data. Replaced "Handbook" with "Product Sheet" in "Kit Contents". Added reference 1; moved other references down. Removed references to hypermethylation and intermediate methylation. Deleted "Dissociation (melting) curve" section in "Data analysis" of "Protocol: EpiTect Methyl II PCR Assay for 2 Genes Using 1 DNA Sample". Deleted description of, and results for, the hypermethylated and intermediately methylated DNA fractions. Reformatted the formulae for calculating "DNA copies resistant (R) to enzyme digestion" and the hypermethylated and unmethylated DNA fractions for internal consistency without changing the final results. Corrected typographical error resulting in missing PCR master mix volumes in Table 4 and Table 10 (M_d column).

QIAGEN reserves the right to occasionally redesign individual assays on the EpiTect Methyl II PCR Arrays for improved performance. This revision history can be accessed by contacting technical support and providing the batch numbers from your arrays.

The EpiTect Methyl II PCR Assays use MethylScreen® technology provided under license from Orion Genomics, LLC.

This product is compatible for use in real-time PCR methodologies, including 5' nuclease processes and dsDNA-binding dye processes claimed in patents owned by Roche Molecular Systems, Inc., F. Hoffmann-La Roche Ltd, and Applera Corporation. No right to perform any of these patented processes is conveyed, expressly, by implication or by estoppel to the purchaser by the purchase of this product. A license to use these patented processes for the purchaser's own internal research accompanies the purchase of certain reagents of Applied Biosystems and other licensed suppliers, or is available from Applied Biosystems. Further information on purchasing licenses may be obtained by contacting the Director of Licensing, Applied Biosystems, 850 Lincoln Centre Drive, Foster City, California 94404, USA.

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