

November 2011

---

# **EpiTect<sup>®</sup> Methyl qPCR Assay Handbook**

For regional DNA methylation analysis using  
MethylScreen<sup>™</sup> technology



---

Sample & Assay Technologies

## **QIAGEN Sample and Assay Technologies**

QIAGEN is the leading provider of innovative sample and assay technologies, enabling the isolation and detection of contents of any biological sample. Our advanced, high-quality products and services ensure success from sample to result.

### **QIAGEN sets standards in:**

- Purification of DNA, RNA, and proteins
- Nucleic acid and protein assays
- microRNA research and RNAi
- Automation of sample and assay technologies

Our mission is to enable you to achieve outstanding success and breakthroughs. For more information, visit [www.qiagen.com](http://www.qiagen.com).

# Contents

<b>Kit Contents</b>	<b>4</b>
<b>Storage</b>	<b>4</b>
<b>Quality Control</b>	<b>4</b>
<b>Product Use Limitations</b>	<b>4</b>
<b>Technical Assistance</b>	<b>5</b>
<b>Safety Information</b>	<b>5</b>
<b>Introduction</b>	<b>6</b>
Principle and procedure	6
<b>Equipment and Reagents to Be Supplied by User</b>	<b>8</b>
<b>Important Notes</b>	<b>9</b>
DNA contamination	9
Genomic DNA preparation	9
Using the EpiTect Methyl DNA Restriction Kit	10
<b>Protocols</b>	
■ <b>EpiTect Methyl qPCR Assay for 5 Genes Using 1 DNA Sample</b>	<b>11</b>
■ <b>EpiTect Methyl qPCR Assay for 2 Genes Using 1 DNA Sample</b>	<b>15</b>
<b>Data Analysis</b>	<b>19</b>
Obtaining raw threshold cycle ( $C_T$ ) values	19
Exporting $C_T$ values	19
Excel-based data analysis template	19
Data quality control	19
Relative amounts of DNA in each methylated fraction	21
Significance of methylation results	21
$\Delta C_T$ data analysis	22
<b>Troubleshooting Guide</b>	<b>25</b>
<b>References</b>	<b>26</b>
<b>Ordering Information</b>	<b>28</b>

## Kit Contents

<b>EpiTect Methyl qPCR Assay</b>	<b>(200)</b>
<b>Catalog no.</b>	<b>335001</b>
Laboratory-verified forward and reverse primers for 200 reactions	1
Handbook	1

## Storage

EpiTect Methyl qPCR Assays are shipped at ambient temperature. Upon receipt, store at –20 °C. If stored under these conditions, EpiTect Methyl qPCR Assays are stable for 6 months after receipt.

## Quality Control

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

## Product Use Limitations

Purchaser agrees that use of this product and data therefrom is limited solely to the purchaser and for only the purchaser's own internal molecular biology research applications ("Permitted Use"), and shall not be re-sold or used for any other purposes (all of which are expressly prohibited), including without limitation diagnostic purposes, uses that could require regulatory approval for diagnostics from an agency of any government or regulatory entity anywhere in the world, diagnosis, prevention, or treatment of disease, and the right to perform commercial services of any kind, including without limitation, reporting the results of purchaser's activities, including without limitation, for a fee or other commercial consideration. Except for the Permitted Use, no rights, titles, or interests in or to any tangible or intangible property rights are conveyed or shall be deemed conveyed by implication, estoppel or otherwise. The performance characteristics of the product other than for the Permitted Use are unknown.

The EpiTect Methyl qPCR 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.

## 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 EpiTect Methyl qPCR Assays 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](http://www.qiagen.com/Support) or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit [www.qiagen.com](http://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](http://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

## Introduction

The EpiTect Methyl qPCR System is an innovative technology enabling fast and accurate detection of CpG island DNA methylation profiles 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. The EpiTect Methyl qPCR Arrays use the MethylScreen™ Technology provided under license from Orion Genomics, LLC.

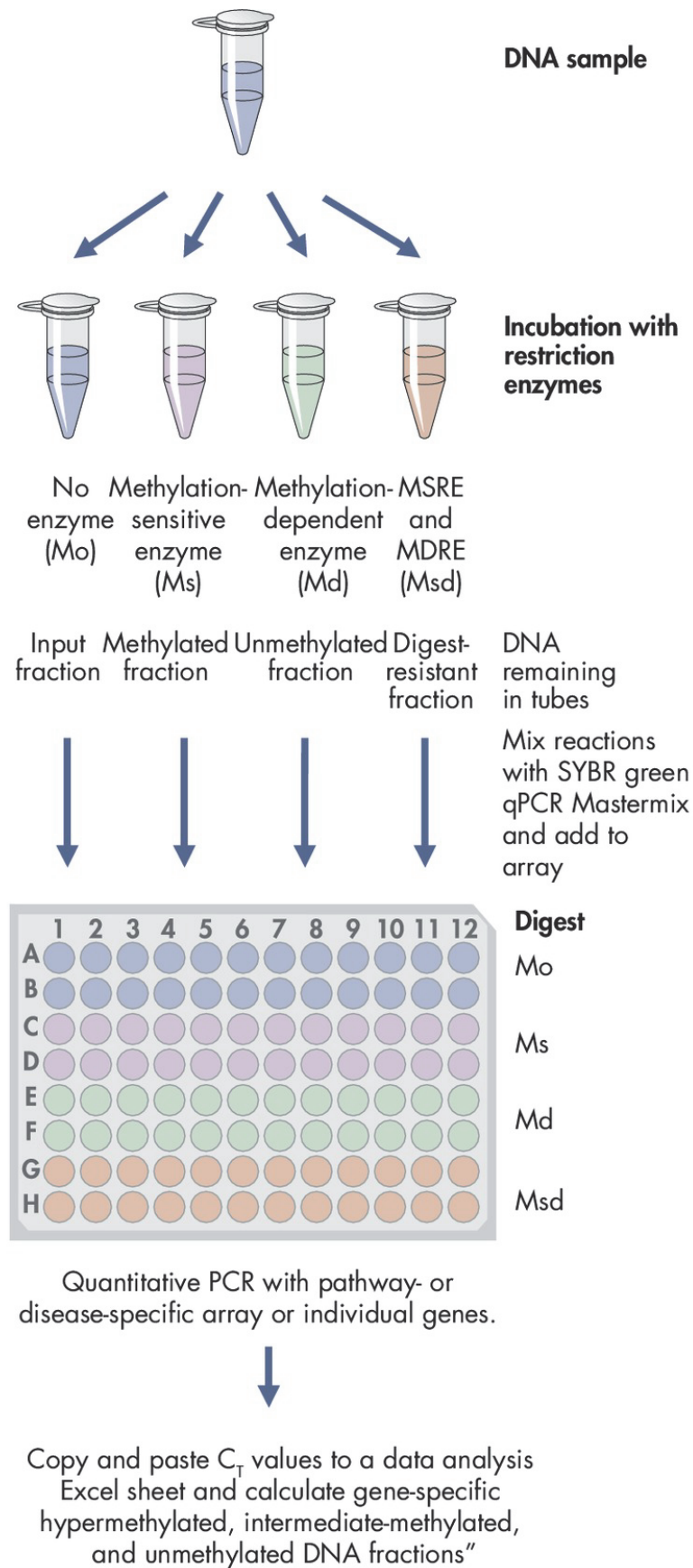
Approximately 60–70% of all human gene promoters overlap with CpG islands — regions with an elevated GC content and a high frequency of CpG dinucleotides. Gene silencing by means of hypermethylation of specific genes promoters is a well-known feature of neoplastic cells and plays an important role in normal cell differentiation and development (1). 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: hypermethylation represses transcription while hypomethylation 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 is based on the detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (2). These enzymes will digest unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA is quantified by real-time PCR in each individual enzyme reaction using primers that flank a promoter (gene) region of interest. The relative fractions of hypermethylated, intermediately methylated, and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest. 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.



**Figure 1. EpiTect Methyl qPCR procedure.**

## 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.

- DNeasy<sup>®</sup> Blood and Tissue Kit (cat. nos. 69504 or 69506) or the AllPrep<sup>®</sup> DNA/RNA Mini Kit (cat. no. 80204) for preparation of DNA
- EpiTect Methyl DNA Restriction Kit (cat. no. 335451).
- Appropriate RT<sup>2</sup> SYBR<sup>®</sup> Green qPCR Mastermix (be sure to select the correct format for the PCR instrument, and size and quantity for the number of samples):
  - RT<sup>2</sup> SYBR Green ROX<sup>™</sup> qPCR Mastermix (cat. nos. 330520, 330522, 330523, 330521, 330529) for all Applied Biosystems<sup>®</sup> and Stratagene<sup>®</sup> instruments, and Eppendorf<sup>®</sup> Mastercycler<sup>®</sup> ep realplex instruments with a ROX filter set
  - RT<sup>2</sup> SYBR Green Fluor qPCR Mastermix (cat. nos. 330510, 330512, 330513, 330511, 330519) for Bio-Rad<sup>®</sup> iCycler<sup>®</sup>, MyiQ<sup>™</sup>, MyiQ2<sup>™</sup>, and iQ<sup>™</sup> 5 instruments
  - RT<sup>2</sup> 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<sup>™</sup>, CFX384<sup>™</sup>, Bio-Rad/MJ Research Opticon 2, and Bio-Rad/MJ Research Chromo4<sup>™</sup>, Roche<sup>®</sup> LightCycler<sup>®</sup> 480 (96- and 384-well)
- Real-time PCR instrument
- Calibrated single- and multi-channel pipets
- RNase-/DNase-free pipet tips and tubes
- RNase-/DNase-free 100  $\mu$ l regular PCR tubes (8- or 12-tube strings)
- Molecular biology grade RNase- and DNase-free water



# Important Notes

## DNA contamination

For reliable results, it is very important to prevent contamination of the EpiTect Methyl qPCR 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 (pipet 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 qPCR 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. For the DNeasy Blood and Tissue Kit, ensure that samples have been treated for the removal of RNA, as 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:  $> 4 \mu\text{g/ml}$

## 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 hypermethylated DNA isolated from samples of heterogeneous cell types, e.g., tumor samples, where heavy non-tumor cell contamination is expected, e.g., blood, stromal cells, etc. 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 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  $-20^{\circ}\text{C}$ . When in use, enzymes should be kept on ice.

# Protocol: EpiTect Methyl qPCR Assay for 5 Genes Using 1 DNA Sample

Be sure to read the Important Notes, page 9, before starting the protocol.

## Restriction digestion

### Procedure

1. Perform the restriction digestions using the EpiTect Methyl DNA Restriction Kit (cat. no. 335451).
2. Prepare a reaction mix without enzymes as indicated in Table 1.

It is recommended to use 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 $\mu$ l
RNase-/DNase-free water	Variable
<b>Final volume</b>	<b>120 <math>\mu</math>l</b>

3. Add RNase-/DNase-free water to make the final volume 120  $\mu$ l. Vortex to thoroughly mix the components and spin down briefly.
4. Set up 4 digestion reactions (Mo, Ms, Md, and Msd) according to Table 2.

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

**Table 2. Restriction digestion**

<b>Component</b>	<b>M<sub>o</sub></b>	<b>M<sub>s</sub></b>	<b>M<sub>d</sub></b>	<b>M<sub>sd</sub></b>
Reaction mix from step 3	28 $\mu$ l	28 $\mu$ l	28 $\mu$ l	28 $\mu$ l
Methylation-sensitive enzyme A	–	1 $\mu$ l	–	1 $\mu$ l
Methylation-dependent enzyme B	–	–	1 $\mu$ l	1 $\mu$ l
RNase-/DNase-free water	2 $\mu$ l	1 $\mu$ l	1 $\mu$ l	–
<b>Final volume</b>	<b>30 <math>\mu</math>l</b>	<b>30 <math>\mu</math>l</b>	<b>30 <math>\mu</math>l</b>	<b>30 <math>\mu</math>l</b>

5. Pipet up and down to gently, but thoroughly mix the components. Spin 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 –20°C. Mix the samples thoroughly by vortexing before use. Spin the samples down briefly and proceed to step 1 of “Setting up the PCR”.

## Setting up the PCR

### Procedure

1. Prepare individual reactions for each of the 4 digestions (M<sub>o</sub>, M<sub>s</sub>, M<sub>d</sub>, and M<sub>sd</sub>) 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 $\mu$ l	12.5 $\mu$ l	12.5 $\mu$ l	12.5 $\mu$ l
PCR primer mix	1.0 $\mu$ l	1.0 $\mu$ l	1.0 $\mu$ l	1.0 $\mu$ l
$M_o$ digest	5 $\mu$ l	–	–	–
$M_s$ digest	–	5 $\mu$ l	–	–
$M_d$ digest	–	–	5 $\mu$ l	–
$M_{sd}$ digest	–	–	–	5 $\mu$ l
RNase-/DNase-free water	6.5 $\mu$ l	6.5 $\mu$ l	6.5 $\mu$ l	6.5 $\mu$ l
<b>Final volume</b>	<b>25 <math>\mu</math>l</b>	<b>25 <math>\mu</math>l</b>	<b>25 <math>\mu</math>l</b>	<b>25 <math>\mu</math>l</b>

**Table 4. PCR setup (384-well plate)**

Component	$M_o$	$M_s$	$M_d$	$M_{sd}$
PCR master mix	5 $\mu$ l	5 $\mu$ l	$\mu$ l	5 $\mu$ l
PCR primer mix	0.4 $\mu$ l	0.4 $\mu$ l	0.4 $\mu$ l	0.4 $\mu$ l
$M_o$ digest	2 $\mu$ l	–	–	–
$M_s$ digest	–	2 $\mu$ l	–	–
$M_d$ digest	–	–	2 $\mu$ l	–
$M_{sd}$ digest	–	–	–	2 $\mu$ l
RNase-/DNase-free water	2.6 $\mu$ l	2.6 $\mu$ l	2.6 $\mu$ l	2.6 $\mu$ l
<b>Final volume</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>

2. Mix tubes well by vortexing, and briefly spin down the contents to the bottom of the tube.
3. Add 25  $\mu$ l of each reaction to the EpiTect Methyl qPCR Assay 96-well plate, as shown in Table 5. For 384-well plates, use 10  $\mu$ l.

**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 <sub>o</sub> digest	A1	A2	A3	A4	A5
M <sub>s</sub> digest	C1	C2	C3	C4	C5
M <sub>d</sub> digest	E1	E2	E3	E4	E5
M <sub>sd</sub> 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

### Procedure

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	30 s	3 cycles
72°C	1 min	
97°C	15 s	40 cycles
72°C	1 min†	
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 19.

# Protocol: EpiTect Methyl qPCR Assay for 2 Genes Using 1 DNA Sample

Be sure to read the Important Notes, page 9, before starting the protocol.

## Restriction digestion

### Procedure

1. Perform the restriction digestions using the EpiTect Methyl DNA Restriction Kit (cat. no. 335451).
2. Prepare a reaction mix without enzymes as indicated in Table 7.

It is recommended to use 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 (250 ng)	Variable
5x Restriction Digestion Buffer	13 $\mu$ l
RNase-/DNase-free water	Variable
<b>Final volume</b>	<b>60 <math>\mu</math>l</b>

3. Add RNase-/DNase-free water to make the final volume 60  $\mu$ l. Vortex to thoroughly mix the components and spin down briefly.
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**

<b>Component</b>	<b>M<sub>o</sub></b>	<b>M<sub>s</sub></b>	<b>M<sub>d</sub></b>	<b>M<sub>sd</sub></b>
Reaction mix from step 3	14 $\mu$ l	14 $\mu$ l	14 $\mu$ l	14 $\mu$ l
Methylation-sensitive enzyme A	–	0.5 $\mu$ l	–	0.5 $\mu$ l
Methylation-dependent enzyme B	–	–	0.5 $\mu$ l	0.5 $\mu$ l
RNase-/DNase-free water	1 $\mu$ l	0.5 $\mu$ l	0.5 $\mu$ l	–
<b>Final volume</b>	<b>15 <math>\mu</math>l</b>	<b>15 <math>\mu</math>l</b>	<b>15 <math>\mu</math>l</b>	<b>15 <math>\mu</math>l</b>

5. Pipet up and down to gently, but thoroughly mix the components. Spin 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 –20°C. Mix the samples thoroughly by vortexing before use. Spin the samples down briefly and proceed to step 1 of “Setting up the PCR”.

## Setting up the PCR

### Procedure

1. Prepare individual reactions for each of the 4 digestions (M<sub>o</sub>, M<sub>s</sub>, M<sub>d</sub>, and M<sub>sd</sub>) 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 $\mu$ l	12.5 $\mu$ l	12.5 $\mu$ l	12.5 $\mu$ l
PCR primer mix	1.0 $\mu$ l	1.0 $\mu$ l	1.0 $\mu$ l	1.0 $\mu$ l
$M_o$ digest	5 $\mu$ l	–	–	–
$M_s$ digest	–	5 $\mu$ l	–	–
$M_d$ digest	–	–	5 $\mu$ l	–
$M_{sd}$ digest	–	–	–	5 $\mu$ l
RNase-/DNase-free water	6.5 $\mu$ l	6.5 $\mu$ l	6.5 $\mu$ l	6.5 $\mu$ l
<b>Final volume</b>	<b>25 <math>\mu</math>l</b>	<b>25 <math>\mu</math>l</b>	<b>25 <math>\mu</math>l</b>	<b>25 <math>\mu</math>l</b>

**Table 10. PCR setup (384-well plate)**

Component	$M_o$	$M_s$	$M_d$	$M_{sd}$
PCR master mix	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l	5 $\mu$ l
PCR primer mix	0.4 $\mu$ l	0.4 $\mu$ l	0.4 $\mu$ l	0.4 $\mu$ l
$M_o$ digest	2 $\mu$ l	–	–	–
$M_s$ digest	–	2 $\mu$ l	–	–
$M_d$ digest	–	–	2 $\mu$ l	–
$M_{sd}$ digest	–	–	–	2 $\mu$ l
RNase-/DNase-free water	2.6 $\mu$ l	2.6 $\mu$ l	2.6 $\mu$ l	2.6 $\mu$ l
<b>Final volume</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>	<b>10 <math>\mu</math>l</b>

2. Mix tubes well by vortexing, and briefly spin down the contents to the bottom of the tube.
3. Add 25  $\mu$ l of each reaction to the EpiTect Methyl qPCR Assay 96-well plate, as shown in Table 11. For 384-well plates, use 10  $\mu$ l.

**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 <sub>o</sub> digest	A1	A2
M <sub>s</sub> digest	C1	C2
M <sub>d</sub> digest	E1	E2
M <sub>sd</sub> 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

### Procedure

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	30 s	3 cycles
72°C	1 min	
97°C	15 s	40 cycles
72°C	1 min†	
According to instrument recommendations	Melting curve segment	

\* Hot-start to activate DNA polymerase.

<sup>†</sup> 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 19.

## 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.

### Excel-based data analysis template

First, download the EpiTect Methyl PCR Array Excel-based data analysis template, which is available at:

[www.sabiosciences.com/dna\\_methylation\\_data\\_analysis.php](http://www.sabiosciences.com/dna_methylation_data_analysis.php).

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 29 cycles, if the recommended amounts of genomic DNA were used.

### Single enzyme digest ( $M_s$ and $M_d$ ) $C_T$ values

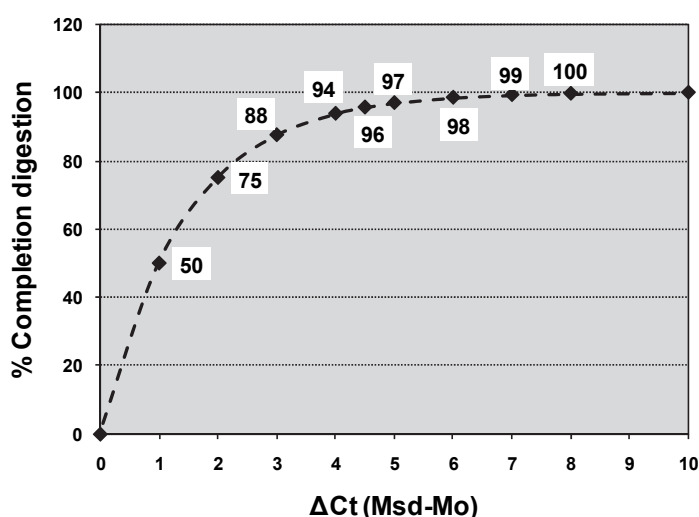
The  $C_T$  values of the  $M_s$  and  $M_d$  digests should be between the values of the mock and double digests, depending on the methylation status of the DNA samples.

## Double digest (Msd) $C_T$ values

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

## Enzyme digestion efficiency

The difference in  $C_T$  values between the double and mock digests should be greater than 2 ( $\Delta C_T [M_{sd} - M_o] > 2$ ) and represents the analytical window (W) of the assay. When W is  $> 2$ , it means that more than 75% of all DNA molecules in the samples were digested; hence the results are reliable and meaningful. See also the "Data QC Report" worksheet in the Excel data analysis template. For every gene, the analytical window (W) values should be  $> 2$  and the R values should be  $< 25\%$ .



## Dissociation (melting) curve

Perform the default melting curve program on the instrument immediately after the cycling program. Generate the first derivative dissociation curve for each well in each plate using the instrument's software. A single well-defined peak should appear in each well. If your instrument does not have a default melting curve program, run the following program instead:

95°C, 1 min; 65°C, 2 min (OPTICS OFF)

65–95°C at 2°C/s (OPTICS ON)

## **Relative amounts of DNA in each methylated fraction**

The “Results” worksheet displays the relative percentage of hypermethylated (HM), intermediately methylated (IM) and unmethylated (UM) DNA in each target genomic DNA sequence. The HM values can be used to generate a graphical representation of the data using our developed Hierarchical Clustering method ([www.sabiosciences.com/dna\\_methylation\\_heatmap.php](http://www.sabiosciences.com/dna_methylation_heatmap.php))

## **Significance of methylation results**

The level of HM methylation considered to be significant (potential positive marker for hypermethylation) must be defined by the researcher. However, the same principles used to define the significance of bisulfite-based sequencing and real-time PCR based methods apply to the EpiTect Methyl qPCR Arrays and qPCR Assays as well. Alternatively, to define whether your results are significant you might take the following into consideration.

### **Percentage of hypermethylated DNA**

In most cases, only hypermethylated promoters will repress gene expression. Therefore, the minimum level of hypermethylation considered to be positive can be set at 10 to 20% (similar to bisulfite-based PCR methods). However, this is dependent on the ratio of target vs. non-target cells present in the sample (i.e. normal cells mixed with cancerous cells). The greater the extent of contamination, the higher the threshold must be set.

### **Comparison between a control and experimental DNA samples**

Such parallel analysis will allow you 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).

### **Hypomethylation (<10%) and high intermediate methylation (>60%)**

Intermediately 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, one should consider measuring the corresponding expression levels and compare those with the expression levels in the appropriate controls.

## $\Delta C_T$ 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:

$$C_{Mo} = 2^{-Ct(M_o)}; C_{Ms} = 2^{-Ct(M_s)}; C_{Md} = 2^{-Ct(M_d)}; C_{Msd} = 2^{-Ct(M_{sd})} \quad (1)$$

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).

Hypermethylated (HM) DNA fraction:

$$F_{HM} = \frac{C_{Ms}}{C_{Mo} - C_{Msd}} = \frac{2^{-Ct(M_s)}}{2^{-Ct(M_o)} - 2^{-Ct(M_{sd})}} \quad (2)$$

Unmethylated (UM) DNA fraction:

$$F_{UM} = \frac{C_{Md}}{C_{Mo} - C_{Msd}} = \frac{2^{-Ct(M_d)}}{2^{-Ct(M_o)} - 2^{-Ct(M_{sd})}} \quad (3)$$

Intermediately methylated (IM) DNA fraction:

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

DNA copies resistant (R) to enzyme digestion:

$$F_R = \frac{C_{Msd}}{C_{Mo}} = \frac{2^{-Ct(M_{sd})}}{2^{-Ct(M_o)}} = 2^{-[Ct(M_{sd}) - Ct(M_o)]} = 2^{-\Delta Ct(M_{sd} - M_o)} \quad (4)$$

Example:

<b>Symbol</b>	<b>M<sub>o</sub></b>	<b>M<sub>s</sub></b>	<b>M<sub>d</sub></b>	<b>M<sub>sd</sub></b>	<b>R</b>	<b>HM</b>	<b>UM</b>	<b>IM</b>
CCNA1	23.16	27.11	24.89	36.51	0.0095%	6.47%	30.15%	63.38%

$$F_{HM} = 2^{-C_T(M_s)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) = 0.0647 \text{ or } 6.47\%$$

$$F_{UM} = 2^{-C_T(M_d)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) = 0.3015 \text{ or } 30.15\%$$

$$F_{IM} = 1 - F_{HM} - F_{UM} = 1 - 0.0647 - 0.3015 = 0.6338 \text{ or } 63.38\%$$

$$F_R = 2^{-C_T(M_{sd} - M_o)} = 2^{-C_T(M_{sd}) - C_T(M_o)} = 2^{-C_T(M_{sd})} / 2^{-C_T(M_o)} = 0.0095\%$$

Methylation-sensitive or methylation-dependent digest C<sub>T</sub> 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<sub>T</sub> value from the mock digest is used to calculate its methylated DNA fraction, whether unmethylated or hypermethylated. The opposite fraction (hypermethylated or unmethylated, respectively) is instead calculated as one minus the determined fraction. The amount of intermediately methylated DNA is then assumed to be negligible.

If  $\Delta C_T(M_s - M_o) < 1.0$  and  $\Delta C_T(M_d - M_o) > 1.0$ , use following formula to calculate the fraction of hypermethylated DNA:

$$F_{HM} = 1 - F_{UM} = 1 - 2^{-C_T(M_d)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) \quad (5)$$

Example:

<b>Symbol</b>	<b>M<sub>o</sub></b>	<b>M<sub>s</sub></b>	<b>M<sub>d</sub></b>	<b>M<sub>sd</sub></b>	<b>R</b>	<b>HM</b>	<b>UM</b>	<b>IM</b>
MGMT	29.95	29.34	36.16	36.50	1.06%	98.64%	1.36%	0.00%

$$F_{UM} = 2^{-C_T(M_d)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) = 0.0136 \text{ or } 1.36\%$$

$$F_{HM} = 1 - F_{UM} = 1 - 0.0136 = 0.9864 \text{ or } 98.64\%$$

$$F_{IM} = 0$$

$$F_R = 2^{-C_T(M_{sd} - M_o)} = 2^{-C_T(M_{sd}) - C_T(M_o)} = 2^{-C_T(M_{sd})} / 2^{-C_T(M_o)} = 1.06\%$$

If  $\Delta C_T (M_d - M_o) < 1.0$  and  $\Delta C_T (M_s - M_o) > 1.0$ , use following formula to calculate the fraction of unmethylated DNA:

$$F_{UM} = 1 - F_{HM} = 1 - 2^{-C_T(M_s)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) \quad (6)$$

Example:

<b>Symbol</b>	<b><math>M_o</math></b>	<b><math>M_s</math></b>	<b><math>M_d</math></b>	<b><math>M_{sd}</math></b>	<b>R</b>	<b>HM</b>	<b>UM</b>	<b>IM</b>
BRCA1	22.96	32.73	23.34	40.00	0.0007%	0.114%	99.886%	0.00%

$$F_{HM} = 2^{-C_T(M_s)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) = 0.00114 \text{ or } 0.114\%$$

$$F_{UM} = 1 - F_{HM} = 1 - 0.00114 = 0.99888 \text{ or } 99.886\%$$

$$F_{IM} = 0$$

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

If both  $\Delta C_T (M_s - M_o)$  and  $\Delta C_T (M_d - M_o)$  are less than 1.0, then the fraction of both hypermethylated and unmethylated DNA are assigned as 50%, while again, the amount of intermediately methylated DNA is negligible.

Example:

<b>Symbol</b>	<b><math>M_o</math></b>	<b><math>M_s</math></b>	<b><math>M_d</math></b>	<b><math>M_{sd}</math></b>	<b>R</b>	<b>HM</b>	<b>UM</b>	<b>IM</b>
SFN	24.03	24.03	24.59	40.00	0.0016%	50.0%	50.0%	0.0%

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

$$F_{IM} = 0$$

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



# 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](http://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](http://www.qiagen.com)).

## Comments and suggestions

---

### Incomplete restriction enzyme digestion: $C_T(M_{sd}) - C_T(M_o) < 2$

- |                                          |                                                                                                                                                                                                                                                                                                                                                                                                                                             |
|------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| a) Poor-quality DNA                      | Repeat the experiment with new DNA samples.                                                                                                                                                                                                                                                                                                                                                                                                 |
| b) Low restriction enzyme activity       | Check that the EpiTect Methyl 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.                                                                                                                                                                                                                                                                |
| 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 use the recommended amount of DNA according to the reaction setup protocol selected.                                                                                                                                                                                                                                                                                                            |
| f) Incorrect incubation conditions       | Incubate for at least 4 hours at 37°C and use the size of tubes recommended in the protocol.<br><br>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       | <p>Be sure to use at least the amount of DNA recommended in the protocol. Use the recommended methods and instruments to determine DNA concentrations.</p> <p>Be sure to include any RNase treatment steps recommended in the procedure of the chosen DNA isolation kit.</p> |
| 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 –20°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 RT <sup>2</sup> SYBR Green qPCR Mastermix.                                                                                                            |

### All 4 digests ( $M_o$ , $M_s$ , $M_d$ , $M_{sd}$ ) $C_T$ values for an individual gene are $\geq 32$

- |                                                                                              |                                                                                                                                                                                                                                                                                     |
|----------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| a) DNA sample may contain a different sequence relative to the most recent NCBI genome build | <p>This may be due to unreported chromosomal abnormalities (insertion or deletions) or single nucleotide polymorphisms (SNPs) that affect the EpiTect Methyl qPCR Assays.</p> <p>Verification may require sequencing of the relevant genomic region in the original DNA sample.</p> |
| b) Homozygous deletions                                                                      | If the $C_T$ values from all 4 digests for an individual gene, but not the majority of genes, are $\geq 32$ , genomic homozygous deletion most likely exists at this locus in the genomic DNA of the original sample.                                                               |

## 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](http://www.qiagen.com/RefDB/search.asp) or contact QIAGEN Technical Services or your local distributor.

### **Cited references**

1. Esteller, M. (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum. Mol. Genet.* **6**, R50.
2. Ordway, JM., et al. (2006) Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets. *Carcinogenesis* **27**, 2409.

## Ordering Information

Product	Contents	Cat. no.
EpiTect Methyl qPCR Assay (200)	Laboratory-verified forward and reverse primers for 200 x 25 µl reactions; 25 µl per primer; total volume: 200 µl	335001
<b>Related products</b>		
EpiTect Methyl Signature qPCR Array (24)	For methylation analysis of 24 genes in a 96-well or 384-well plate format 2, 12, or 24 x 96; 4 x 384	335211
EpiTect Methyl Complete qPCR Array (96)	For methylation analysis of 96 genes in a 96-well or 384-well plate format 4 x 96; 2, 12, or 24 x 384	335221
EpiTect Methyl Custom qPCR Array	For methylation analysis of customer-selected genes in a 96-well or 384-well plate format	335111
EpiTect Methyl 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	335451
EpiTect Methyl qPCR Assay (200)	Laboratory-verified forward and reverse primers for 200 x 25 µl reactions; 25 µl per primer; total volume: 200 µl	335001
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.

<b>Product</b>	<b>Contents</b>	<b>Cat. no.</b>
RT <sup>2</sup> 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); all other cyclers	330500
RT <sup>2</sup> 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
RT <sup>2</sup> 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
RT <sup>2</sup> PCR Array Loading Reservoir	5 ml capacity reservoir for convenient sample loading on PCR arrays	338162

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](http://www.qiagen.com) or can be requested from QIAGEN Technical Services or your local distributor.

\* Larger kit sizes available; please inquire.

## Notes

Trademarks: QIAGEN®, AllPrep®, DNeasy®, EpiTect®, RNeasy® (QIAGEN Group); Applied Biosystems®, StepOnePlus™, ROX™, SYBR® (Life Technologies Corporation); Bio-Rad®, iCycler®, Chromo4™, CFX96™, DNA Engine Opticon®, CFX384™, iQ™, MyiQ™, MyiQ2™ (Bio-Rad Laboratories, Inc.); Eppendorf®, Mastercycler® (Eppendorf AG); MethylScreen™ (Orion Genomics, LLC); Roche®, LightCycler® (Roche Group); SmartCycler® (Cepheid); Stratagene®, Mx3005P®, Mx3000P®, Mx4000® (Stratagene); Excel®, Microsoft® (Microsoft Corporation). Registered names, trademarks, etc. used in this document, even when not specifically marked as such, are not to be considered unprotected by law.

#### Limited License Agreement

Use of this product signifies the agreement of any purchaser or user of the EpiTect Methyl qPCR Assay to the following terms:

1. The EpiTect Methyl qPCR Assay may be used solely in accordance with the *EpiTect Methyl qPCR Assay Handbook* and for use with components contained in the Kit only. QIAGEN grants no license under any of its intellectual property to use or incorporate the enclosed components of this Kit with any components not included within this Kit except as described in the *EpiTect Methyl qPCR Assay Handbook* and additional protocols available at [www.qiagen.com](http://www.qiagen.com).
2. Other than expressly stated licenses, QIAGEN makes no warranty that this Kit and/or its use(s) do not infringe the rights of third-parties.
3. This Kit and its components are licensed for one-time use and may not be reused, refurbished, or resold.
4. QIAGEN specifically disclaims any other licenses, expressed or implied other than those expressly stated.
5. The purchaser and user of the Kit agree not to take or permit anyone else to take any steps that could lead to or facilitate any acts prohibited above. QIAGEN may enforce the prohibitions of this Limited License Agreement in any Court, and shall recover all its investigative and Court costs, including attorney fees, in any action to enforce this Limited License Agreement or any of its intellectual property rights relating to the Kit and/or its components.

For updated license terms, see [www.qiagen.com](http://www.qiagen.com).

The EpiTect Methyl qPCR 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 Applied Biosystems. 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.

© 2011 QIAGEN, all rights reserved.

[www.qiagen.com](http://www.qiagen.com)

**Australia** ■ Orders 1-800-243-800 ■ Fax 03-9840-9888 ■ Technical 1-800-243-066

**Austria** ■ Orders 0800-28-10-10 ■ Fax 0800-28-10-19 ■ Technical 0800-28-10-11

**Belgium** ■ Orders 0800-79612 ■ Fax 0800-79611 ■ Technical 0800-79556

**Brazil** ■ Orders 0800-557779 ■ Fax 55-11-5079-4001 ■ Technical 0800-557779

**Canada** ■ Orders 800-572-9613 ■ Fax 800-713-5951 ■ Technical 800-DNA-PREP (800-362-7737)

**China** ■ Orders 86-21-3865-3865 ■ Fax 86-21-3865-3965 ■ Technical 800-988-0325

**Denmark** ■ Orders 80-885945 ■ Fax 80-885944 ■ Technical 80-885942

**Finland** ■ Orders 0800-914416 ■ Fax 0800-914415 ■ Technical 0800-914413

**France** ■ Orders 01-60-920-926 ■ Fax 01-60-920-925 ■ Technical 01-60-920-930 ■ Offers 01-60-920-928

**Germany** ■ Orders 02103-29-12000 ■ Fax 02103-29-22000 ■ Technical 02103-29-12400

**Hong Kong** ■ Orders 800 933 965 ■ Fax 800 930 439 ■ Technical 800 930 425

**Ireland** ■ Orders 1800 555 049 ■ Fax 1800 555 048 ■ Technical 1800 555 061

**Italy** ■ Orders 800-789-544 ■ Fax 02-334304-826 ■ Technical 800-787980

**Japan** ■ Telephone 03-6890-7300 ■ Fax 03-5547-0818 ■ Technical 03-6890-7300

**Korea (South)** ■ Orders 080-000-7146 ■ Fax 02-2626-5703 ■ Technical 080-000-7145

**Luxembourg** ■ Orders 8002-2076 ■ Fax 8002-2073 ■ Technical 8002-2067

**Mexico** ■ Orders 01-800-7742-639 ■ Fax 01-800-1122-330 ■ Technical 01-800-7742-436

**The Netherlands** ■ Orders 0800-0229592 ■ Fax 0800-0229593 ■ Technical 0800-0229602

**Norway** ■ Orders 800-18859 ■ Fax 800-18817 ■ Technical 800-18712

**Singapore** ■ Orders 1800-742-4362 ■ Fax 65-6854-8184 ■ Technical 1800-742-4368

**Spain** ■ Orders 91-630-7050 ■ Fax 91-630-5145 ■ Technical 91-630-7050

**Sweden** ■ Orders 020-790282 ■ Fax 020-790582 ■ Technical 020-798328

**Switzerland** ■ Orders 055-254-22-11 ■ Fax 055-254-22-13 ■ Technical 055-254-22-12

**UK** ■ Orders 01293-422-911 ■ Fax 01293-422-922 ■ Technical 01293-422-999

**USA** ■ Orders 800-426-8157 ■ Fax 800-718-2056 ■ Technical 800-DNA-PREP (800-362-7737)

