ZYMO RESEARCH

Non-Bisulfite
Method

OneStep qMethyl™ Kit

Bisulfite-free procedure for rapid, locus-specific DNA methylation assessment

Highlights

- Single step, real-time PCR procedure for bisulfite-free determination of DNA methylation status
- Includes reagents and controls for quantitative detection and reliable performance
- Ideal for rapid screening of single and multi-locus DNA methylation

D5310

Scan with your smart-phone camera to view the online protocol/video.

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Product Contents

OneStep qMethyl™ Kit	D5310 Full Kit
2X Test Reaction PreMix$_1$	0.5 ml
2X Reference Reaction PreMix$_1$	0.5 ml
DNase/RNase-free Water	1 ml
MGMT Primers I & II (10 µM each)$_2$	40 µl
Human Methylated & Non-methylated DNA Standards (4 ng/µl each)$_2$	40 µl
Instruction Manual	1

1 Store the Test Reaction PreMix and the Reference Reaction PreMix at -20°C (-80°C for long-term storage) upon arrival.
2 Store the *MGMT* Primers and the Standards at -20°C (-80°C for long-term storage) upon arrival.
The PreMixes in this kit contain SYTO 9® dye for analysis using real-time PCR. The OneStep qMethyl™-Lite product (Cat. No. D5311) omits this dye. The "Lite" version allows real-time PCR to be performed with other fluorescent dyes or molecular probes of the researcher's choosing.

Introduction

Epigenetic modifications are regarded as fundamental to the regulation of gene expression. DNA methylation is one such modification that plays crucial roles in widespread biological phenomena including the regulation of gene activity, gene imprinting, embryonic development and X-chromosome inactivation in higher organisms. Current methods used to evaluate DNA methylation including bisulfite sequencing, Methylation Specific PCR (MSP), HPLC, and Methylated DNA Immunoprecipitation (MeDIP) have proven too costly, time consuming, or inappropriate for the investigation of large numbers of regions. **The OneStep qMethyl™ Kit** from Zymo Research provides a simple, straightforward, and bisulfite-free procedure for rapid, region-specific DNA methylation assessment. Simply add DNA into the appropriate reaction mix then quantitate via real-time PCR… *OneStep*!

Simple OneStep DNA Methylation Determination
Unlike conventional qAMP procedures that contain multiple steps, **the OneStep qMethyl**™ method integrates the workflow into a simple, single step reaction. This minimizes errors that can occur during setup and the likelihood of contamination while allowing for rapid and accurate DNA methylation level detection.

Overview of Procedure

The **OneStep qMethyl™ Kit** is used for the detection of region-specific DNA methylation via the selective amplification of methylated cytosines in CpG dinucleotide context. This is accomplished by splitting any DNA to be tested into two parts: a "*Test Reaction*" and a "*Reference Reaction*" (see Figure 1 below). DNA in the *Test Reaction* is digested with Methylation Sensitive Restriction Enzymes (MSREs) while DNA in the *Reference Reaction* is not. The DNA from both samples is then amplified using real-time PCR in the presence of SYTO® 9 fluorescent dye and then quantitated. Cycle threshold (Ct) values for Test and Reference DNA samples will vary depending on methylation status, with large Ct differences most characteristic of non-methylated DNA..

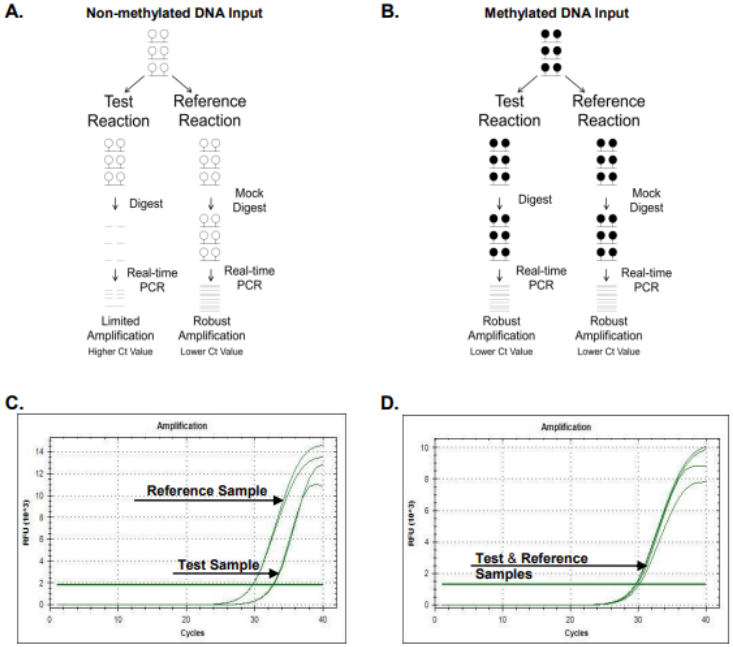


Figure 1. Schematics A and B (above) illustrate the sample workflow of Non-methylated DNA and Methylated DNAs. In both cases the DNA is divided in two parts; a Test Reaction and a Reference Reaction. Test Reaction samples are MSRE digested while the Reference Reaction samples are not (mock digested). Following digestion, DNA from both samples is used for real-time PCR. The white lollipops in the image represent unmethylated cytosines and black lollipops methylated cytosines in CpG dinucleotide context. Following real-time PCR,amplification plots (C and D) demonstrate non-methylated DNA exhibits large differences in the Ct values for Test and Reference Reactions (C) while highly methylated DNA samples exhibit little difference (D).

Considerations for Experimental Design

A. DNA Quality

Input DNA processed using the **OneStep qMethyl™** procedure should be high quality and suitable for use in restriction enzyme (i.e., MSRE) digestion. If input sample purity is in question, it is recommended to use the **Genomic DNA Clean & Concentrator ™** (Cat. Nos. D4010, D4011, Zymo Research Corp.) For FFPE samples, we recommend the **Quick-DNA™ FFPE MiniPrep** (Cat No. D3067, Zymo Research Corp.) for purifying high quality, intact dsDNA.

B. Input DNA and Reaction Volume

Each reaction mixture for the **OneStep qMethyl™** procedure (page 5) is optimized for 20 ng input DNA. For each sample, 5 µl DNA (4 ng/µl) should be added to bring the final reaction volume to 20 µl (i.e., 1 ng/µl final concentration). Input DNA should be diluted in water or "modified" TE containing a low concentration of EDTA (10 mM Tris pH 8.0-8.5. 0.1 mM EDTA).

C. Duplicate Sampling

Precise Ct value determination is critical for accurate quantification of DNA methylation by real-time PCR using the **OneStep qMethyl™** procedure. Therefore, it is recommended to set up each Test Reaction and Reference Reaction measurement in duplicate to ensure accurate, non-biased data collection.

D. Primer Design and PCR Amplicons

Primers should span a DNA region that is 120 bp to 350 bp and contains at least two (2) MSRE sites. See Appendix I (page 9) for a list of the MSREs featured in the **OneStep qMethyl™** Kit. Primers can be designed using conventional procedures.

E. Human Methylated & Non-methylated DNA Standards

It is critical to include the Human Methylated & Non-methylated DNA Standards (in duplicate) with the control ***MGMT* primers** to validate the accuracy of **the OneStep qMethyl™** procedure for determining methylation percentage.

Protocol

The following protocol illustrates the use of the **OneStep qMethyl™** procedure for DNA methylation detection at a single region[1]. This allows for the processing of up to 22 DNA samples (in duplicate)[2] using a 96-well real-time PCR plate. The provided **Human Methylated & Non-methylated DNA Standards**[3] should be processed along with the samples for the purpose of validating the combined MSRE digestion/real-time PCR step (see Appendix III, page 11). The following should serve as a guideline when setting up your own experiment. *The format is also compatible with thin-walled PCR tubes and tube strips.*

I. Preparation of Test Reaction and Reference Reaction Mixtures[4]

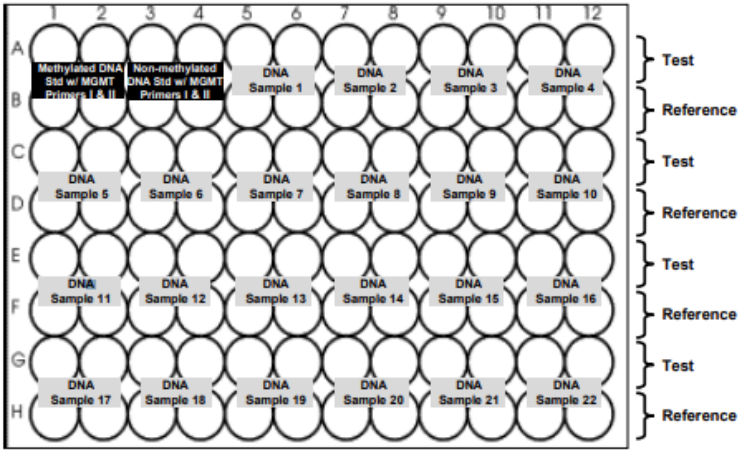
1. For each DNA sample (and Standard) to be analyzed, setup (on ice) both **a Test Reaction** and a **Reference Reaction** mixture.

Test Reaction (per well)	**Volume (µl)**
2X Test Reaction PreMix (contains MSREs)	10
10 µM **MGMT Primer I & II**	2
DNase/RNase-free Water	3
TOTAL VOLUME	15

Reference Reaction (per well)	**Volume (µl)**
2X Reference Reaction PreMix	10
10 µM **MGMT Primer I & II**	2
DNase/RNase-free Water	3
TOTAL VOLUME	15

Make a **Test** and **Reference Reaction** master mix to accommodate the 22 DNA samples and the **Methylated & Non-methylated DNA Standards**. Add 15 µl of **Test** and **Reference Reaction** master mix to the wells of a 96-well real-time PCR plate (not provided) according to the diagram on the following page.

1 See Appendix II to use the OneStep qMethyl™-PCR procedure for simultaneous methylation level determination in multiple regions.
2 Alternatively, if not performed in duplicate, 44 individual DNA samples can be processed.
3 Enough of the Human Methylated & Nonmethylated DNA Standards is provided for a single run, 96-well analysis.
4 The final concentration of MgCl2 in the Reference Reaction & Test Reaction mixtures is 3.75 mM. The final concentrations of primers will need to be optimized. Final concentrations shown here are at 500 nM. Final primer concentrations range between 250 nM to 800nM



2. Add 5 µl (20 ng) of the appropriate DNA sample to those designated wells (*in duplicate*) as shown in the diagram.

3. Seal the plate with sealing tape or a similar device[1]. Transfer to a 96-well real-time PCR instrument (e.g., Applied Biosystems 7500 Series, Roche LightCycler™, BioRad CFX96™, Illumina Eco™ Real-Time PCR System, or similar). Proceed with Step II below.

II. OneStep MSRE Digestion/Real-Time PCR

Reaction conditions for the combined MSRE Digestion/Real-Time PCR processes have been optimized for direct input, digestion, and real-time amplification of DNA samples. However, annealing temperature and elongation time may need to be optimized depending on the design of the primers. The parameters below are for the supplied ***MGMT*[2] primers** and should be used as a guideline when setting up your own experiment. Typically, between 40-45 cycles is recommended for the amplification of most DNA templates.

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1 To eliminate bubbles that may be present within the wells, spin down the plate prior to conducting real-time PCR (Step II).
2 Refer to Appendix III for detailed information regarding the **Human Methylated** and **Non-methylated DNA Standard** and ***MGMT* primers**.

1. Set the real-time PCR instrument to excitation and emission wavelengths of (~) 465 nm and 510 nm, respectively.

Note: a setting that is compatible with SYBRgreen® should be compatible with the SYTO 9® dye in this kit.

2. Perform real-time PCR using the following parameters.

Step	Temperature	Time (hr:min:sec)	
1 – MSRE Digestion	30 °C	12:00:00	
2	95 °C	00:08:00	
3	97 °C	00:02:00	
4	97 °C	00:00:20	} Steps 4-5, Repeat 40 cycles
5[1, 2]	58 °C	00:01:00	
6 – Hold	4 °C	> 5 minutes	

III. Data Analysis

The methylation level for any amplified region can be determined using the following equation:

$$\text{Percent Methylation} = 100 \times 2^{-\Delta Ct}$$

where ΔCt = the average Ct value from the **Test Reaction** minus the average Ct values from the **Reference Reaction**.

1 A melt analysis can be performed after Step 5 prior to the Hold Step. The melt analysis is recommended for the detection of non-specific product and primer dimer formation.

2 The annealing temperature time with may vary with user designed primers and the size of the amplicon. Therefore, it may be necessary to adjust and optimize these conditions.

Example:

The table (below) represents actual real-time PCR data from the **OneStep qMethyl™** procedure using the **Human Non-methylated DNA Standard** and ***MGMT* primers** (performed in duplicate).

Ct values of **Test Reaction**	Ct values of **Reference Reaction**
33.83	29.76
33.81	29.81

To determine the methylation level of the **Human Non-methylated DNA standard**,

1. Calculate the average Ct values for **Test** and **Reference** Reactions.

Ct values of **Test Reaction**	Ct values of **Reference Reaction**
33.82	29.79

2. Determine the ΔCt by subtracting the average Ct value of **Reference Reaction** from the average Ct value of **Test Reaction**.

   $$\Delta Ct = 33.82 - 29.79 = 4.03$$

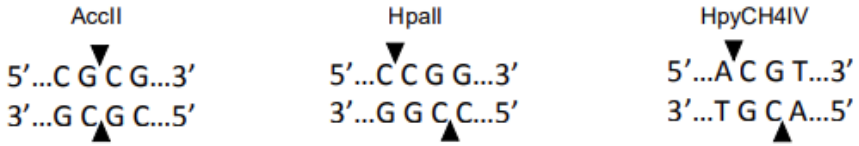
3. Substitute the ΔCt value into the equation: $100 \times 2^{-\Delta Ct}$

   $$100 \times 2^{-4.03} = 6\%$$

   Using this equation, the methylation level of the **Human Non-methylated DNA Standard** is determined to be 6 % at the region spanned by the ***MGMT* primers**. The actual value is ~5% as determined by bisulfite sequencing methods. Due to the low background level of methylation in Zymo's non-methylated standard cell line, the results of the calculation are within the expected limit of methylation detection as illustrated in Appendix III, page 11.

Appendix I Methylation Sensitive Restriction Enzymes (MSREs) Featured in the **OneStep qMethyl™** Procedure

It is important to consider the consensus sequences of those MSREs (below) when designing primers to any particular DNA region, since any particular region should contain at least two (2) MSRE sites for accurate methylation assessment using the **OneStep qMethyl™** procedure. A mix of the following MSREs are present in the Test Reaction Premix:



Appendix II Using the **OneStep qMethyl™** Procedure for Methylation Level Detection for Multiple Regions

The protocol on page 5 illustrates the use of the **OneStep qMethyl™** procedure for the evaluation of methylation levels within a single region from multiple DNA samples. However, methylation determination within multiple regions of a particular DNA sample is often required. For methylation assessment of 22 different regions in a DNA sample, the following example is provided.

Protocol for Methylation Detection Levels for Multiple Regions

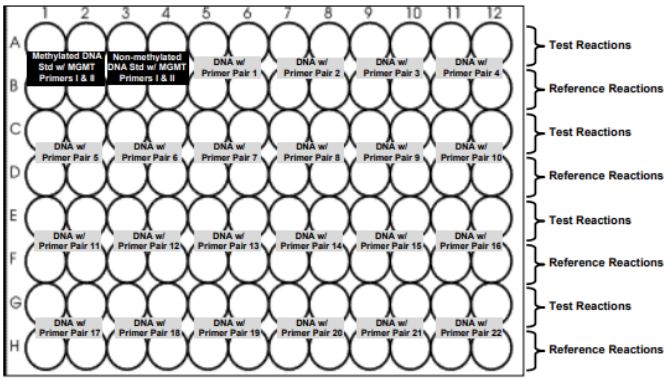
1. For each region to be analyzed, setup (on ice) both a Test Reaction and Reference Reaction mixture[1] .

Test Reaction (per well)	**Volume (µl)**
2X Test Reaction PreMix (contains MSREs)	10
DNA (or **Methylated & Non-methylated DNA Standard**) (4 ng/µl)	5
DNase/RNase-free Water	3
TOTAL VOLUME	18

1 The final concentration of $MgCl_2$ in the Reference Reaction & Test Reaction mixtures is 3.75 mM.

Reference Reaction (per well)	Volume (µl)
2X Reference Reaction PreMix DNA (or **Methylated & Non-methylated DNA Standard**) (4 ng/µl) **DNase/RNase-free Water**	10 5 3
TOTAL VOLUME	18

Make a **Test** and **Reference Reaction** master mix to accommodate duplicate sampling of 22 samples and **the Methylated & Non-methylated DNA Standards**. Add 18 µl of **Test** and **Reference Reaction** master mix to the wells of a 96-well real-time PCR plate according to the diagram below:



2. Dilute primers to a final concentration of 10 µM in **DNase/RNase-free Water** and then add 2 µl of the appropriate primers (pre-mixed) to those designated wells (*in duplicate*) as shown in the diagram[1]. Continue with Step 3 in the standard protocol (page 6).

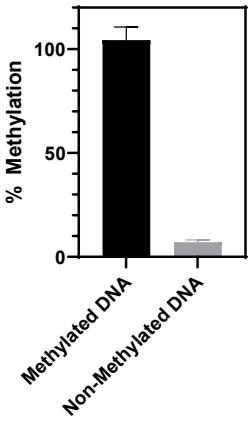
1 The final concentration of primers will need to be optimized. Final concentrations shown here are at 500 nM. Final primer concentrations range between 250 nM to 800 nM.

Appendix III The **Human Methylated & Non-methylated DNA Standards** and ***MGMT*** **Primer Set**

This kit contains **Human Methylated & Non-methylated DNA Standards** with an ***MGMT*** **primer set** for validating the **OneStep qMethyl™** system. Enough material is provided for a single run, 96-well analysis. The **Human Methylated DNA** has been enzymatically methylated at all cytosine positions comprising CG dinucleotides using M.Sss I methyltransferase. The **Human Non-methylated DNA** was purified from cells containing genetic knockouts of both DNMT1 and DNMT3b DNA methyltransferases and has a low level of DNA methylation (~5%).

Below is the observed lot-to-lot variation in Methylation levels of **the Human Methylated & Non-Methylated DNA Standards** when using the **OneStep qMethyl Kit**.

Methylation level spanned by the MGMT primers



Product Specifications

I. **Human Non-Methylated DNA Standard**
Source: DNA purified from HCT116 DKO cells [DNMT1 (-/-) / DNMT3b (-/-)].
Concentration: 4 ng/µl in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Storage: -20 ºC.

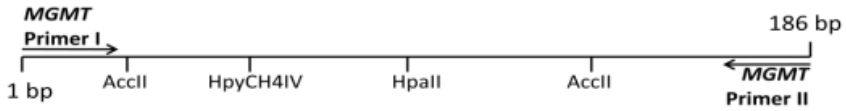
II. **Human Methylated DNA Standard**
Source: DNA purified from HCT116 DKO cells [enzymatically methylated by M.SssI Methyltransferase (EC 2.1.1.37)]. Concentration: 4 ng/µl in buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Storage: -20 ºC.

III. ***MGMT* Primers Set**
Concentration: 10 µM in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).
Volume: 20 µl of each primer.
Storage: -20 ºC.

MGMT Primer I: 5′ - GGT GTG AAA ACT TTG AAG GA - 3′
MGMT Primer II: 5′ - CAC TAT TCA AAT TCC AAC CC - 3′

IV. The expected amplicon for the **Human Methylated & Non-methylated DNA Standard** with ***MGMT* I & II primers** (underlined) is ~180 bp and contains four (4) MSRE sites (see sequence and diagram below) [1].

-1121 gggtgtgaaa actttgaagg aaaccgcgtc aagagcctgg
-1161 ctgattgtta atatcacgtt aactcagagg gccaggatac
-1201 ttgcccagac ccggagtctg cctgcaagta gcagaggaga
-1251 gctggccttg ctctgccgcg tgtctttctt cctgggccct
-1291 ctgtctcggg ttggaatttg aatagtg



MGMT Region

1 At least 2-4 MSRE sites should be contained in an amplicon ranging from 150 to 350 bp long. This size is range is often necessary to obtain the minimum number of MSRE sites.

Ordering Information

Product Description	Catalog No.	Size
OneStep qMethyl™ Kit	D5310	1 x 96 well
OneStep qMethyl-Lite	D5311	1 x 96 well

Individual Kit Components	Catalog No.	Amount
2X Test Reaction PreMix	D5310-1	0.5 ml
2X Reference Reaction PreMix	D5310-2	0.5 ml
DNase/RNase-free Water	W1001-1	1 ml

Notes

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Notes

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Integrity of kit components is guaranteed for up to one year from date of purchase.
Reagents are routinely tested on a lot-to-lot basis to ensure they provide the highest performance and reliability.

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