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ZYMO RESEARCH

OneStep PLUS qMethyl™ PCR Kit

Enzymatic Real-Time PCR for the detection of DNA methylation

Highlights

- No bisulfite conversion needed: from DNA sample to results in just one step.
- Accurate quantification of DNA methylation levels at genomic regions of choice.
- Simple reaction assembly with pre-mixed reagents and controls.
- Maximum flexibility: compatible with both SYBR® Green and TaqMan® probes-based assays.

Catalog Numbers:
D5312

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

tech@zymoresearch.com www.zymoresearch.com Toll Free: (888) 882-9682

Table of Contents

Product Contents

OneStep PLUS qMethyl™ PCR Kit	D5312 48 reactions	Storage Temperature
2X Test PreMix	0.5 ml	-20°C
2X Reference PreMix	0.5 ml	-20°C
Fluor Dye (20X)	100 µl	-20°C
qMethyl Control Primer I & II (10 µM each)	40 µl	-20°C
Human Methylated DNA Standards (4 ng/µl)	40 µl	-20°C
Human Non-methylated DNA Standards (4 ng/µl)	40 µl	-20°C
DNase/RNase Free Water	1 ml	-20°C

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, carcinogenesis, embryonic development, and X-chromosome inactivation. Current methods used to evaluate DNA methylation including bisulfite sequencing, Methylation Specific PCR (MSP), HPLC, and Methylated DNA Immunoprecipitation (MeDIP) have proven costly, laborious, time consuming, or not adequate for the screening of large numbers of regions. In addition, several of these methods rely on bisulfite conversion of the DNA which cause significant sample degradation. The OneStep PLUS qMethyl™ PCR Kit from Zymo Research provides a simple and bisulfite conversion-free procedure for the precise quantification of DNA methylation at genomic regions of choice. Simply add DNA into the pre-assembled reaction mix and quantitate via Real-Time PCR… *OneStep*!

Simple OneStep Assessment of DNA Methylation

Unlike conventional procedures that require multiple steps, the **OneStep PLUS qMethyl™ PCR** method comes in a ready-to-use, single-step reaction. This simplifies the procedure and minimizes the chance of introducing errors during the setup while allowing for rapid and accurate measurement of DNA methylation level.

Overview of Procedure

The OneStep PLUS qMethyl™ PCR Kit is intended for the quantification of DNA methylation at custom-selected genomic regions. The same DNA sample is analyzed in parallel with the Test PreMix and the Reference PreMix. The Test PreMix contains enzymes that selectively digest unmethylated DNA while leaving methylated DNA intact. Only methylated DNA will be amplified in the "*Test Reaction."* The Reference PreMix does not contain those enzymes, therefore both methylated and unmethylated DNA are amplified in the "*Reference Reaction."* The difference in Cycle threshold (Ct) values between the Reference and Test reactions is used to calculate the percentage of DNA methylation at the selected genomic region. A schematic overview of the procedure is represented in Figure 1 below.

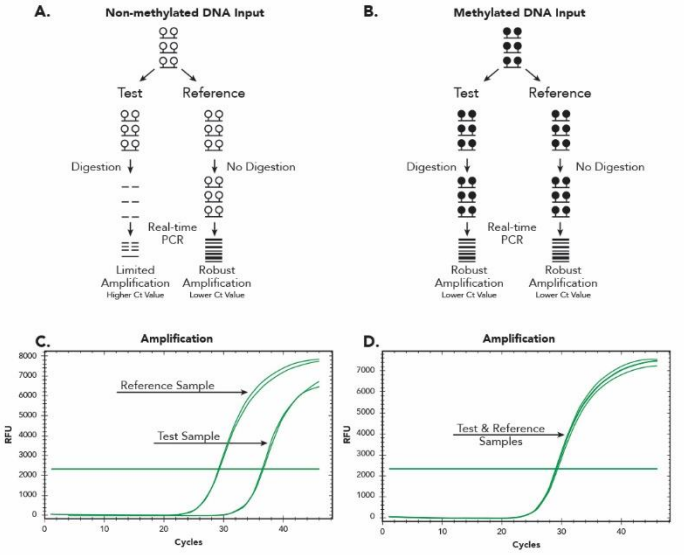


Figure 1. A and B are the schematic representation of OneStep PLUS qMethyl™ PCR test for Non-methylated DNA and Methylated DNA, respectively. Non-methylated and methylated CpG sites are represented by white and black circles, respectively. In both schemes, the DNA is tested in two reactions: a Test Reaction, in which non-methylated DNA is digested, and a Reference Reaction, in which no digestion occurs. Quantitative PCR is carried out immediately after the digestion step. C and D are examples of amplification curves obtained from Human Non-Methylated & Methylated DNA Standards, respectively. The higher Ct values for the Test Reaction compared to the Reference reaction in figure C indicates that the non-methylated DNA fraction has been degraded during the digestion step.

Considerations for Experimental Design

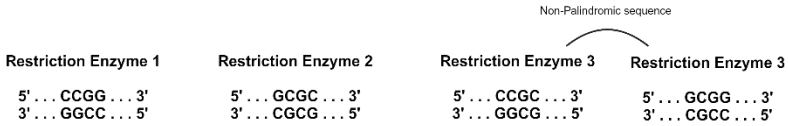
A. DNA Quality and Input

Input DNA processed using the OneStep PLUS qMethyl™ PCR procedure should be high quality and suitable for use in restriction enzyme digestion and Real-Time PCR assays. If input sample purity is in question, it is recommended to use the **Genomic DNA Clean & Concentrator™** (Cat. Nos. D4010, D4011).

Input range is 5 ng – 1 µg, however it is recommended to use 20 ng of DNA per reaction.

B. Primer Design and PCR Amplicons

Primers should span a DNA region that is 70 bp to 350 bp long. It is important to consider the consensus sequences of the Methyl Specific Restriction Enzymes (MSREs, see below) when designing primers to any particular DNA region. The region should contain at least three (3) MSRE sites for accurate methylation assessment. A mix of the following MSREs is present in the Test Premix:



Avoid including restriction sites in the primer sequence.

Primers can be designed using conventional procedures. The PCR set-up in the protocol is optimized for the annealing temperature of the qMethyl Control Primers. The annealing temperature may vary with user designed primers. Therefore, it may be necessary to adjust and optimize this annealing temperature step of the protocol (Page 7, Step 5 of the PCR parameters).

C. Human Methylated & Non-methylated DNA Standards

It is recommended to test in each experiment the Human Methylated & Non-methylated DNA Standards with either the qMethyl Control Primers or with the user designed primer. This verifies the performance of the reagents used and correct reaction setup.

Protocol

The following protocol illustrates the use of the OneStep PLUS qMethyl™ PCR procedure for DNA methylation detection of a *single genomic region*[1]. The provided Human Methylated & Non-methylated DNA Standards[2] should be tested along with the samples to verify the performance of the reagents used and correct reaction setup (see Appendix II, page 11). The following protocol is a guideline for setting up your own experiment; some optimization may be required for your specific regions and primers.

I. Preparation of Test Reaction and Reference Reaction Mixtures

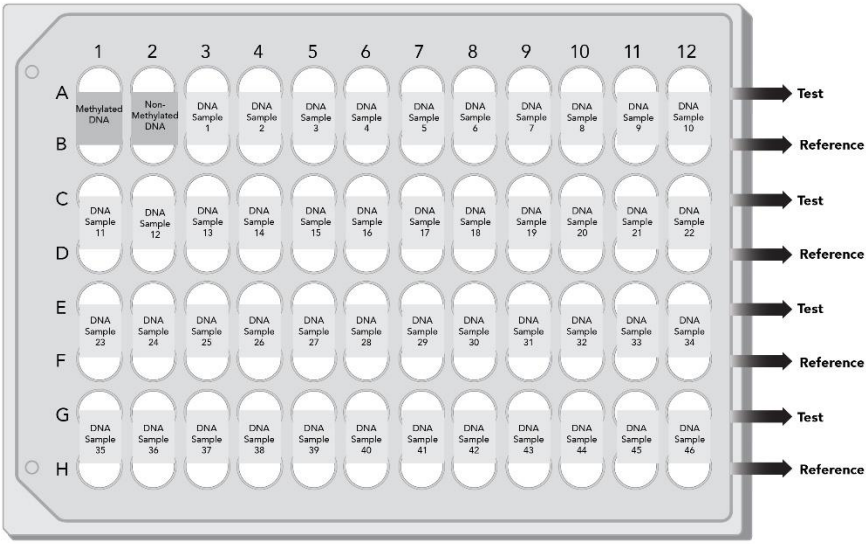
1. For each DNA sample (and Standard) to be analyzed, set up (on ice) a **Test Reaction** and **Reference Reaction** mixture as described below.

   The volumes indicated are for a single reaction; for the analysis of multiple samples, multiply the volume of each component by the number of the samples to test[3].

Test Reaction (per well)	Volume (µl)
2X Test PreMix	10
Custom Primer (forward and reverse) (10 µM each)	2
Fluor Dye (20X)	1
DNase/RNase-free Water	2
TOTAL VOLUME	15

Reference Reaction (per well)	Volume (µl)
2X Reference PreMix	10
Custom Primer (forward and reverse) (10 µM each)	2
Fluor Dye (20X)	1
DNase/RNase-free Water	2
TOTAL VOLUME	15

1 See Appendix I to use the OneStep PLUS qMethyl™ PCR procedure for the determination of DNA methylation of multiple genomic regions.
2 The amount of Human Methylated & Non-methylated DNA Standards provided is sufficient for four runs.
3 It is recommended to include 1-2 extra reactions when assembling the **Test** and **Reference Reaction** master mixes to ensure enough reagent during plating.



2. Transfer 15 µl of **Test** and **Reference Reaction** master mix to PCR wells according to the suggested diagram above.

3. Add 5 µl (5 ng - 1µg) of the appropriate DNA sample to the appropriate wells[4].

4. Seal the plate (or tubes) with sealing film suitable for Real-Time PCR analysis[5]. Transfer to a Real-Time PCR instrument (e.g., BioRad CFX96™ or similar). Proceed with Step II.

4 Set-up in the diagram illustrates sampling as a singlet. Users can set-up **Test** and **Reference** reaction measurement for each sample in duplicate to ensure accurate, non-biased data collection.

5 To eliminate bubbles that may be present within the wells, spin down the plate prior to conducting real-time PCR (Step II).

II. Digestion & Real-Time PCR

The reaction conditions for the combined Digestion & Real-Time PCR have been optimized for the **qMethyl Control Primer I & II** provided with the kit[6]. The annealing temperature may need to be optimized depending on the design of the primers.

1. Select the SYBR® Green/FAM filter on the Real-Time PCR instrument.

2. Set-up the following Real-Time PCR parameters. [7,8,9]

Step	Temperature	Time (hr:min:sec)	
1 –Digestion	30 °C	12:00:00	
2	95 °C	00:08:00	
3	97 °C	00:02:00	
4	97 °C	00:00:20	Repeat steps 4-5 for total of 40 cycles
5	58 °C	00:01:00	
6 – Hold *(optional)*	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 Ct value from the **Test Reaction** minus the Ct values from the **Reference Reaction**.

6 Refer to Appendix II for detailed information regarding the Human Methylated and Non-methylated DNA Standard and qMethyl Control primers.
7 A minimum of 2 hours is required for the digestion step, but 12 hours is optimal.
8 The annealing temperature (Step 5 of the PCR parameters) may vary with user designed primers and the size of the amplicon. Therefore, it may be necessary to adjust and optimize this step.
9 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 products and primer dimer formation.

Example:

The table (below) is an example of Real-Time PCR data from the OneStep PLUS qMethyl™ PCR procedure using the **Human Non-methylated DNA Standard** and **qMethyl Control primers**.

Sample	Ct values of **Test Reaction**	Ct values of **Reference Reaction**
Human Non-methylated DNA Standard	33.92	27.52

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

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

   $\Delta Ct = 33.92 - 27.52 = 6.4$

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

   $100 \times 2^{-6.4} = 1.184\%$

   Using this equation, the methylation level of the **Human Non-methylated DNA Standard** is determined to be 1.184 % at the region spanned by the qMethyl Control primers.

The actual value of the region is determined to be ~1-5% as determined by bisulfite sequencing methods. Due to the low background level of methylation in HCT116 DKO cell line, the results of the calculation are within the expected limit of methylation detection.

Appendix I

Using the OneStep PLUS qMethyl™ PCR Procedure for Methylation Level Detection for Multiple Regions

The protocol on page 5 illustrates the use of the OneStep PLUS qMethyl™ PCR 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 up to 48 different regions in a DNA sample, the following example is provided.

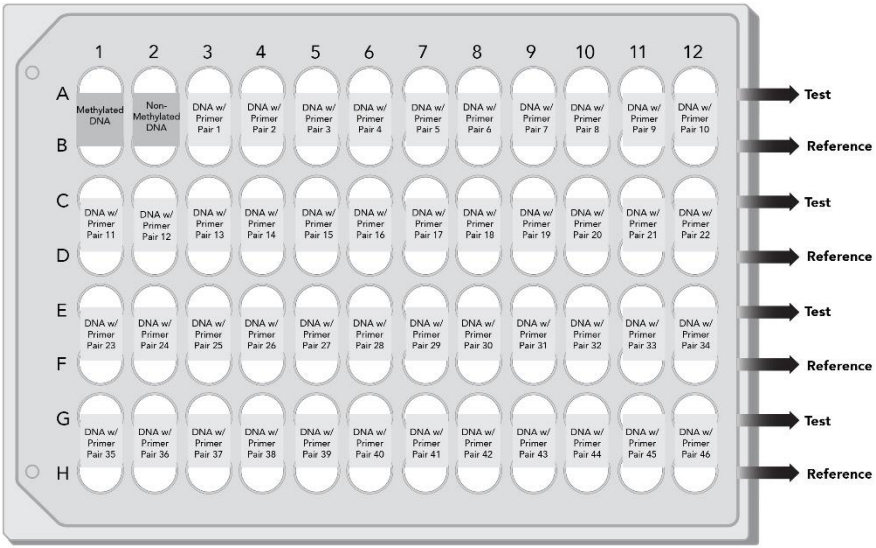
Protocol for Methylation Detection Levels for Multiple Regions

1. For each region to be analyzed, set up (on ice) both a **Test Reaction** and **Reference Reaction** mixture. The volumes indicated are for a single reaction; for the analysis of multiple samples multiply the volume of each component by the number of the samples to test.

Test Reaction (per well)	Volume (µl)
2X Test PreMix (contains MSREs)	10
DNA (or Methylated & Non-Methylated Standard) (4ng/µl)	5
Fluor Dye (20X)	1
DNase/RNase-free Water	2
TOTAL VOLUME	18

Reference Reaction (per well)	Volume (µl)
2X Reference PreMix	10
DNA (or Methylated & Non-Methylated Standard) (4ng/µl)	5
Fluor Dye (20X)	1
DNase/RNase-free Water	2
TOTAL VOLUME	18

Transfer 18 µl of **Test** and **Reference Reaction** master mix to PCR wells according to the suggested diagram on the following page:



2. Dilute primers to a final concentration of 10 µM in **DNase/RNase-free Water.** Then, transfer 2 µl of the appropriate primers to those designated wells as shown in the diagram[10]. Continue with Step 4 in the standard protocol (page 6, Section I, Step 4).

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

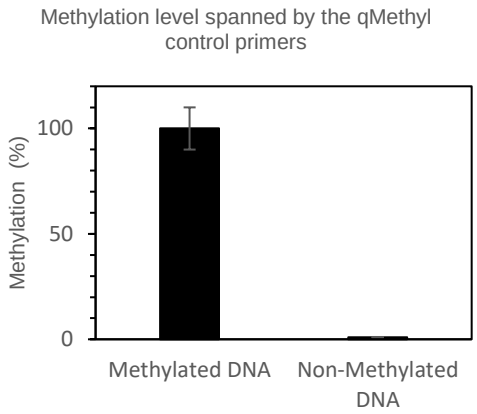
Appendix II

The Human Methylated & Non-methylated DNA Standards and qMethyl Control Primer Set

This kit contains Human Methylated & Non-methylated DNA Standards and qMethyl Control Primer Set for validating the OneStep PLUS qMethyl™ PCR system. The qMethyl Control Primers can be used to evaluate the integrity of the reagents and workflow, or to evaluate the performance of the user designed primers (as a comparison).

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%). The Human Methylated DNA was derived by enzymatically methylating the Non-methylated DNA, so all CpG dinucleotides are methylated.

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



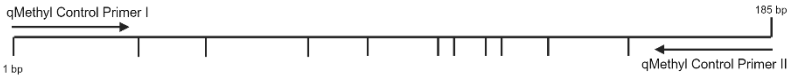
Product Specifications

- **Human Methylated DNA Standard**
  Source: DNA purified from HCT116 DKO cells [enzymatically methylated].
  Concentration: 4 ng/µl in TE buffer.

- **Human Non-Methylated DNA Standard**
  Source: DNA purified from HCT116 DKO cells [DNMT1 (-/-) / DNMT3b (-/-)].
  Concentration: 4 ng/µl in TE buffer.

- **qMethyl Control Primer I & II**
  Concentration: Pooled primers at 10 µM each in TE buffer.
  Forward: 5′ - CCT GCA CCC AGG TTT CCA TT
  Reverse: 5′ - CTC AAT GAG CTC AGG CTC CC

The expected amplicon for the Human Methylated & Non-methylated DNA Standard with qMethyl Control Primer I & II (underlined) is ~180 bp and contains ten (10) MSRE sites (see figure below).



Ordering Information

Product Description	Catalog No.	Size
OneStep PLUS qMethyl™ PCR Kit	D5312	48 reactions
Human Methylated & Non-methylated DNA Set	D5014	5 µg/20 µl
Genomic DNA Clean & Concentrator-10	D4010	25 Preps

Notes

Notes

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