Enzo Life Sciences

Product Manual

BioArray™ 5-hmC Methylation Kit

Catalog #: ENZ-45011

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

5-Hydroxymethylcytosine (^{5hm}C), also known as the “6th base”, is a newly discovered epigenetic modification. Although it was first identified in bacteriophages, its role was thought to be limited to protecting the phage genome from host induced restriction endonucleases[1]. Interestingly, 5-hydroxymethylcytosine has recently been found in embryonic stem cells, the brain, as well as numerous other organs[2,3]. Even though its presence has been confirmed, its biological role remains elusive. It has been proposed that 5-hydroxymethylcytosine may play a role in DNA demethylation (5-methylcytosine) or be involved in another layer of gene expression regulation[3].

To date, studies of 5-hydroxymethylcytosine has been limited to global quantification or characterization studies. The reason being is that the “standard” for 5-methylcytosine detection, bisulfite DNA analysis, is unable to effectively distinguish 5-methylcytosine from 5-hydroxymethylcytosine.

The **BioArray™ 5-hmC Methylation Kit** allows for sequence specific detection of 5-hydroxymethylcytosine within DNA using a simple and efficient reaction setup.

ASSAY PRINCIPLE

The highly specific and robust **5-hmC Glucosyltransferase** enzyme, 5-hydroxymethylcytosine in DNA is specifically tagged with a glucose moiety yielding a modified base, glucosyl-5-hydroxymethylcytosine (Figure 1).

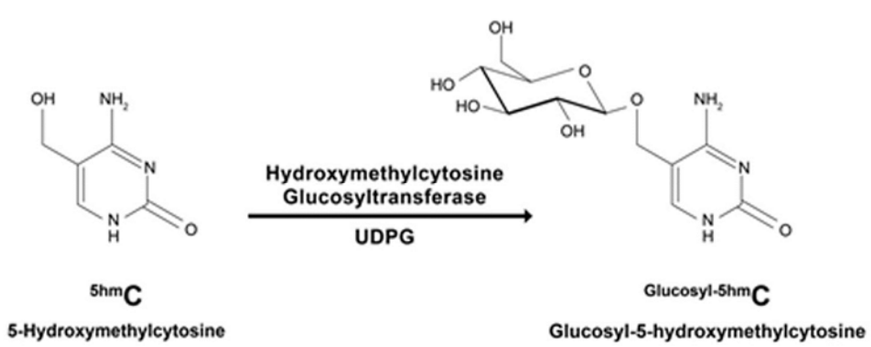


Figure 1: 5-hmC Glucosyltransferase transfers a glucose moiety from uridine diphosphoglucose (UDPG) onto preexisting 5-hydroxymethylcytosines within DNA.

After glucosylation of 5-hydroxymethylcytosine, digestion of DNA with "glucosyl-5-hydroxymethylcytosine sensitive" restriction endonucleases (GSREs) allow differentiation of 5-methylcytosine from 5-hydroxymethylcytosine according to the context of a GSRE's recognition sequence (**see Table 1**). GSREs can efficiently digest DNA when cytosine, 5-methylcytosine, or 5-hydroxymethylcytosine is within their recognition sequence. However, if 5-hydroxymethylcytosine is glucosylated (i.e., glucosyl-5-hydroxymethylcytosine), GSREs can no longer digest the DNA (**Figure 2**). Exploitation of this sensitivity to glucosyl-5-hydroxymethylcytosine facilitates the effective detection 5-hydroxymethylcytosine using a number of downstream applications (e.g., qPCR, NextGen Sequencing, Southern blotting, array, etc.). qPCR is recommended to assess locus-specific context of 5-hydroxymethylcytosine within the DNA Treatment of DNA containing ^{5hm}C with 5-hmC Glucosyltransferase specifically adds a glucose moiety yielding glucosyl-5-hydroxymethylcytosine. Subsequent digestion with GSREs (see Table 1 below) will specifically cleave DNA having cytosine, 5-methylcytosine, or 5-hydroxymethylcytosine in the recognition sequence. Glucosyl-5-hydroxymethylcytosine in the DNA will remain uncleaved.

GSRE	Recognition Sequence
MspI (included w/ this kit)	C*C*GG
GlaI	GCGC
Csp6I	GTAC
HaeIII	GGC*C*
Taq$^{\alpha}$I	TCGA
MboI	GATC
McrBC	$R^{m}C(N_{40\text{-}3000})R^{m}C$

Table 1: $^{Glucosyl\text{-}5hm}C$ Sensitive Restriction Endonucleases (GSREs). Detection of 5-hmC with MspI and HaeIII is *only* at the indicated "C" position (underlined and italicized).
Taq$^{\alpha}$I and MboI display incomplete sensitivity to $^{Glucosyl\text{-}5hm}C$. Titration of the enzyme amount and incubation time will be required to yield optimal results.

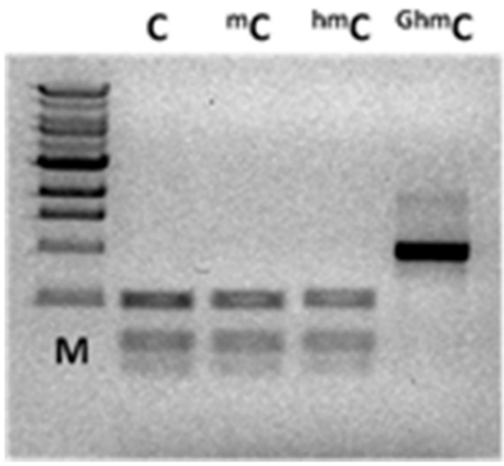


Figure 2: Glucosyltransferase (5-hmC) inhibits cleavage of 5hmC DNA by the GSRE Csp6I. DNA with all cytosines as: unmodified (C), 5-methylcytosine (^{m}C), 5-hydroxymethylcytosine (^{hm}C) or glucosyl-5-hydroxymethylcytosine (^{Ghm}C) was digested with 4 units of Csp6I for 2 hours at 37°C and resolved in a 0.8% w/v agarose/TAE/EtBr gel. "M" is 1kb DNA ladder.

After processing DNA with the BioArray™5-hmC Methylation Kit subsequent analysis of ^{5hm}C sites can be achieved with qPCR, ultra-deep sequencing, Southern blotting, or microarray.

Additional information on the assay principle and applications can be found in the Appendix I Section of the manual.

MATERIALS SUPPLIED

BioArray™ 5-hmC Detection Kit	Supply	Storage Temp.
5-hmC Glucosyltransferase (5-hmC)*	100 µL (2 units/µl)	-20 °C
10X 5-hmC Reaction Buffer	1 mL	-20 °C
10X UDPG (Uridine Diphosphoglucose)	600 µL (1mM)	-20 °C
MspI Restriction Enzyme*	150 µL (10 units/µl)	-20 °C
5-hmC Control DNA(1)	300 ng	-20 °C
qPCR Primers 1 (2)	1 Unit (20 µM)	-20 °C
qPCR Primers 2 (2)	1 Unit (20 µM)	-20 °C
5-DNA Clean up Concentrator Kit ** • **DNA Binding** • **DNA Wash Buffer** • **DNA Elution Buffer** • **Spin Column** • **Collection Tube**	50 preps	RT

***AVOID MULTIPLE FREE THAWING AND LONG TERM STORAGE -80ºC.**

****ADDITIONAL COMPONENTS PROVIDED**

(1) **5-hmC Control DNA** – A 90bp DNA standard used for qPCR. [10ng/µl] in storage buffer (10mM Tris-HCl; 0.1mM EDTA; pH 8.0) supplied in 30µl volume. See **Appendix** for more details.

(2) **qPCR Primers1 and 2** – Control primers specific to 5-hmC Control DNA (above) validated for qPCR. See **Appendix** for detailed information.

STORAGE

Store reagents suggested conditions. *RT-Room Temperature

STABILITY

12 months upon receipt

PRECAUTIONS

1. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately.
2. Since some reagents are irritants, wear protective gloves and eye protection. Should be used by trained professionals
3. Interpretation of the results is the sole responsibility of the user.

Reagants require separate storage conditions.

REAGENT PREPARATION

NOTE: Reagents stored at freezing temperatures should be removed prior to experiment and thawed. Reagents should be briefly centrifuged to ensure contents are fully collected.

5-hmC Control DNA – A 90bp DNA standard used for qPCR. [10ng/µl] in storage buffer (10mM Tris-HCl; 0.1mM EDTA; Ph 8.0) supplied in 30µl volume. See **Appendix** for more details.

qPCR Primers 1 and 2 – Control primers specific to **5-hmC Control DNA** (above) validated for qPCR. See **Appendix** for detailed information.

METHODS AND PROCEDURES

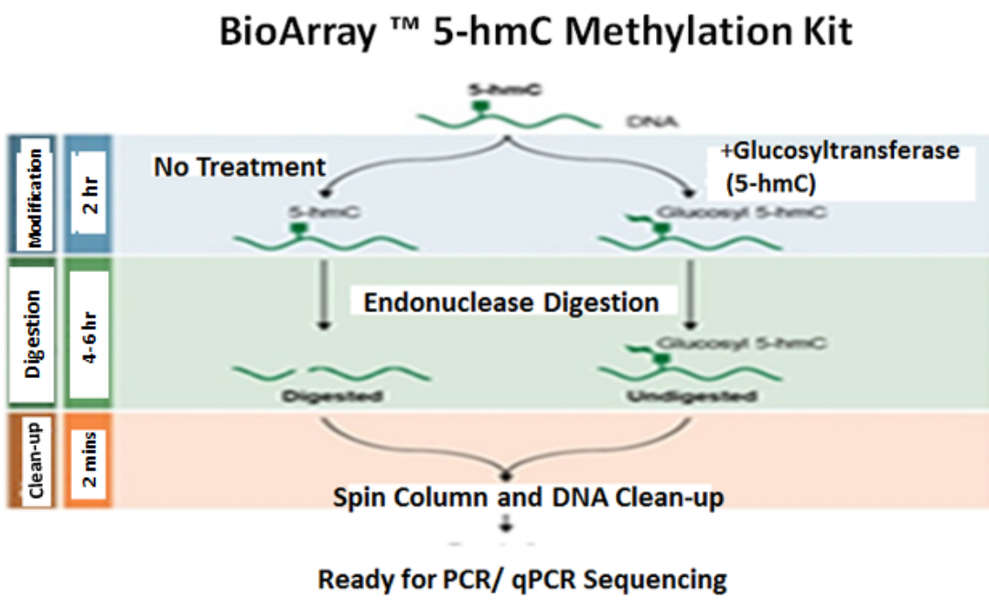


Figure 3: Overall work flow of the BioArray™ 5-hmC Methylation Kit (^{5hm}C detection in DNA). DNA samples are first modified via glucosylation of ^{5hm}C within DNA and then digested with MspI. A low elution spin column is featured for clean-up of ultra-pure DNA which is then used in qPCR for locus-specific analysis of ^{5hm}C.

PROTOCOL

Note: the procedure is also compatible with other GSREs (please see Table 1 and **Appendix)**, and other downstream molecular analysis procedures including NextGen sequencing, Southern blotting, array, etc.

A. Glucosylation Reaction

Divide equal amounts of each DNA to be tested into each of the two separate reaction setups (**1** & **2**) below. Reaction mixtures containing 5-hmC Control DNA should be set up in parallel (separately) to test DNAs.

1. + Glucoslyation Reaction Setup

Test DNA [100-500 ng] or 5-hmC Control DNA [20-50 ng]	x µl
10X 5-hmC GT Reaction Buffer	5 µl
10X UDPG [1mM]	5 µl
5-hmC Glucosyltransferase (2 units/µl)	2 µl
ddH2O	x µl
Total	**50 µl**

Note: To ensure the glucosylation reaction is complete, it is recommended that: **a.** A higher enzyme to DNA ratio is used (e.g., use 4 units of 5-hmC Glucosyltransferase:1µg of DNA). **b.** Extend incubation times to ≥2 hours at 37°C.

2. Glucoslyation Reaction Setup

Test DNA [100-500 ng] or 5-hmC Control DNA [20-50 ng]	x µl
10X 5-hmC Reaction Buffer	5 µl
10X UDPG [1mM]	5 µl
5-hmC GT Enzyme (2 units/µl)	--
ddH2O	x µl
Total	**50 µl**

Incubate reaction mixtures with DNA at 37°C for ≥ 2 hours.

B. Digestion w/ MspI

Note: Alternative GSREs (e.g., Csp6I, HaeIII) can be used in lieu of or together with MspI. GlaI is a methylation dependent GSRE. Test DNA *must* be fully methylated with CpG and GpC DNA methyltransferase *in vitro* prior to cleavage with GlaI. GlaI is compatible with the *single tube reaction format,* but cannot be used in conjunction with other GSREs.

1. Following the incubation from Section **A 1 or 2,** add 30 units of MspI enzyme directly to each (+Glucosylation and -Glucosylation) reaction mixture.
2. Incubate at 37°C for ≥ 4 hours.

Note: Extended digestion times and/or additional MspI may be necessary for complete digestion of DNA which is important for subsequent analysis.

C. Spin Column Clean-up

1. Review the components of the **5- DNA Clean up Concentrator Kit** that is included within this kit.
2. Add a 5:1 ratio of **DNA Binding Buffer** to the reaction mixtures (e.g., 250 µl **DNA Binding Buffer** to each 50 µl reaction mixture from Step B)
3. Transfer mixture to the provided **Spin Column** that is placed in the Collection Tube. Centrifuge for 30 seconds (between *10,000-16,000 xg*). Discard the flow-through.
4. Add 200µL DNA Wash Buffer to the column. Centrifuge for 30 seconds (between *10,000-16,000 xg*). Repeat the wash step.
5. Add ≥6µL DNA Elution Buffer directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a new 1.5mL microcentrifuge tube and centrifuge (between *10,000-16,000 xg*) for 30 seconds to elute the DNA.
   Note: Elution from the column is dependent on pH and temperature. DNA elution Buffer consists of 10mM Tris-HCL pH 8.5, 0.1 mM EDTA. If water is used, make sure that the pH is >6.0. Wait one minute to improve the yield of larger DNA.
6. Following elution of the DNA, qPCR is recommended for locus-specific 5-hmC analysis (see **Step D** below).

D. Locus-Specific 5-hmC Identification via qPCR

Note: For effective analysis of 5-hmC by qPCR, primers must be designed to flank the GSRE recognition site of interest as shown in **Figure 4.**

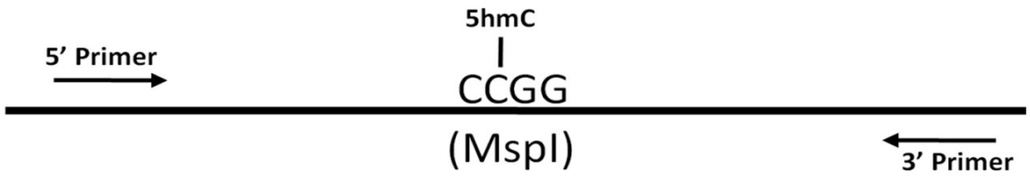
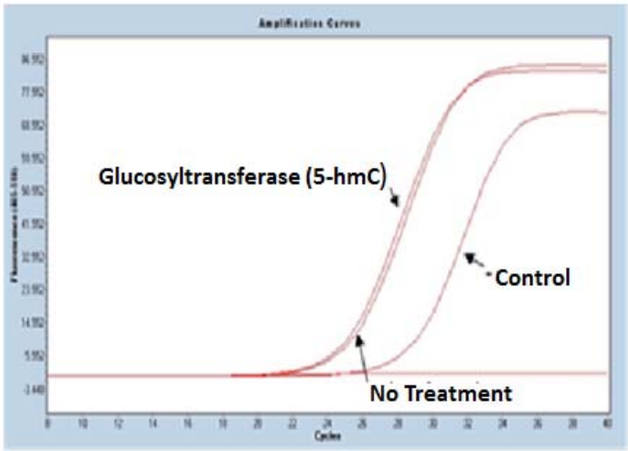


Figure 4: Design of primers for the area of interest to complete qPCR.

Note 2: Each locus requires a proper control for accurate detection of 5-hmC by qPCR as shown in **Figure 5**. This can be achieved by adding test DNA to the qPCR that has not been processed according to **steps A-C** (above).



MSP1 Digestion qPCR

Sample	Cp Value
no treatment	25.1
- control	28.5
+5-hmC Glucosyltransferase	24.9

Figure 5: Detection of 5-hMC by qPCR. The "no treatment" DNA will establish the level (i.e., Cp value) that is representative of complete hydroxymethylation at the interrogation site. Conversely, the "- control" (unglucosylated sample) will indicate a level of no hydroxymethylation at the site. The "+Glucosyltransferase (5-hmC)" i.e glucosylated sample, Cp value should be somewhere between the two, i.e., "no treatment" and "- control". This will depend on the extent of hydroxymethylation at the interrogation site. *GSRE digested unglucoslyated DNA (i.e., "- control") will always yield a signal albeit at a higher Cp value than the other samples.*

Appendix I

5-hmC Control DNA

The **5-hmC Control DNA** is provided for gauging glucosylation and GSRE reaction efficiencies via qPCR. Incorporated into the **5-hmC Control DNA** are 3 GSRE sites (MspI, HaeIII and Csp6I) which can be used for digestion. The **5-hmC Control DNA** is a 90 bp, double stranded DNA that is partially hemi-5-hydroxymethylated. All cytosine residues in the sequence below that are highlighted in grey (i.e., top strand only) are hydroxymethylated.

Sequence of **5-hmC Control DNA**:

1 90

caaggatcgctcgcggctcttaGGC**CGGtaactgtctgcagctctgagGTAC**gcatggattgtaggcgccgccctataccttgtctgcct

gttcctagcgagcgccgagaatCCGGCCattgacagacgtcgagactcCATGcgtacctaacatccgcggcgggatatggaacagacgga

Note: All cytosines in the highlighted sequence are 5-hydroxymethylcytosine. MspI (CCGG), HaeIII (GGCC) and Csp6I (GTAC) sites are capitalized and underlined. MspI and HaeIII sites overlap.

Sequence of qPCR Primers:

qPCR Primer 1	5′- caaggatcgctcgcggctctta -3′
qPCR Primer 2	5′- aggcagacaaggtatagggcg -3′

Appendix II

Glucosylation of 5-hmC in DNA

Use the protocol below to completely glucosylate 5-hmC in DNA (without the GSRE digestion and qPCR analysis steps given in the standard protocol of the manual)

Note: *This can be used for the quantification of global ^{5hm}C via isotope labeling using Uridine Diphosphate Glucose [Glucose-$^{14}C(U)$].*

Glucosylation Reaction Setup

DNA [100-500 ng]	10 µl
10X 5-hmC GT Reaction Buffer	5 µl
10X UDPG [1mM]	5 µl
5-hmC Glucosyltransferase Enzyme (2 units/µl)	2 µl
ddH2O	28 µl
Total	**50 µl**

5-hmC in DNA should be fully glucosylated after incubating the reaction at 37°C for ≥ 2 hours. DNA can be recovered using the clean-up procedure in the protocol (**Step C in Methods and Procedures**)

Appendix III

Digestion of Glucosylated-5-hmC DNA using other GSREs

For the GSREs MspI, GlaI, Csp6I, and HaeIII, glucosylation followed by digestion can be performed (i.e. single tube reaction format). However, TaqI, MboI, and McrBC are incompatible with the *single tube reaction format* and the protocol given below should be used with these enzymes instead.

Step 1 – Glucosylation Reaction

Divide equal amounts of each DNA to be tested into each of the two separate reaction setups (**A** & **B**) below. Reaction mixtures containing **5-hmC Control DNA** should be set up in parallel (separately) to test DNAs.

A. + Glucoslyation Reaction Setup

Test DNA [100-500 ng] or 5-hmC Control DNA [20-50 ng]	x µl
10X 5-hmC Reaction Buffer	5 µl
10X UDPG [1mM]	5 µl
5-hmC Glucosyltransferase (5-hmC) (2 units/µl)	2 µl
ddH2O	x µl
Total	**50 µl**

B. Glucoslyation Reaction Setup

Test DNA [100-500 ng] or 5-hmC Control DNA [20-50 ng]	x µl
10X 5-hmC Reaction Buffer	5 µl
10X UDPG [1mM]	5 µl
5-hmC Glucosyltransferase (5-hmC) (2 units/µl)	--
ddH2O	x µl
Total	**50 µl**

Incubate reaction mixtures with DNA at 37°C for ≥ 2 hours.

Step 2 – Spin Column Clean-up

a. Add a 5:1 ratio of **DNA Binding Buffer** to the reaction mixtures (e.g., 250µl **DNA Binding Buffer** to each 50µl reaction mixture).
b. Transfer mixture to the provided **Spin Column** that is placed in the Collection Tube. Centrifuge for 30 seconds (between *10,000-16,000 xg*). Discard the flow-through.
c. Add 200µL DNA Wash Buffer to the column. Centrifuge for 30 seconds (between *10,000-16,000 xg*). Repeat the wash step.
d. Add ≥6µL DNA Elution Buffer directly to the column matrix and incubate at room temperature for one minute. Transfer the column to a new 1.5mL microcentrifuge tube and centrifuge (between *10,000-16,000 xg*) for 30 seconds to elute the DNA.

Step 3 – GSRE Digestion

Eluted DNA from the previous step should be digested with GSRE following supplier's recommended conditions.

Note: *Over digestion of DNA is recommended. Extended digestion times and/or additional units of GSRE may be necessary for complete digestion of DNA which is important for subsequent analysis.*

Step 4 – Spin Column Clean-up

Repeat Step 2 from this Appendix Section. Following elution of the DNA, qPCR is recommended for locus-specific 5-hmC analysis.

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