EPIGENETICS

EpiMark®
5-hmC and 5-mC Analysis Kit

Instruction Manual

NEB #E3317S
20 reactions

NEW ENGLAND
BioLabs® Inc.
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Kit Components:

Each kit contains sufficient reagents for 20 reactions. All kit components should be stored at –20°C. They can be stored under these conditions for up to 12 months without any reduction in performance.

T4 Phage β-glucosyltransferase (10 units/µl)	60 µl
UDP-Glucose (25X) (2 mM)	250 µl
MspI (100 units/µl)	40 µl
HpaII (50 units/µl)	40 µl
Proteinase K (20 mg/ml)	120 µl
Unmodified Control DNA (0.1 ng/µl)	50 µl
5-mC Control DNA (0.1 ng/µl)	50 µl
5-hmC Control DNA (0.1 ng/µl)	50 µl
Forward and Reverse Control Primer Mix (10 µM each)	40 µl
NEBuffer 4 (10X)	1.5 ml

Control DNA Sequence

5´-CAGTGAAGTTGGCAGACTGAGCCAGGTCCCACAGATGCAGTGACCGGAGT

CATTGCCAAACTCTGCAGGAGAGCAAGGGCTGTCTATAGGTGGCAAGTCA-3´

Control DNA substrates are synthetic 100 bp double stranded fragments containing a single MspI/HpaII site (CCGG). The three fragments are identical except for modification of the internal C in this site.

FW Primer Sequence

5´- CA GTG AAG TTG GCA GAC TGA GC -3´

REV Primer Sequence

5´- CTG ACT TGC CAC CTA TAG ACA GC -3´

Required Materials Not Included:

Heat block or water bath (suitable for temperatures of 37°C, 40°C and 95°C)

PCR materials:

- Locus-specific primers, flanking a CCGG site of interest
- DNA polymerase for PCR
- Nucleotides for PCR
- PCR Thermal Cycler (for endpoint experiments, option IIIa)
- Real-time PCR cycler (for quantitative experiments, option IIIb)

0.2 ml strip tubes and caps for PCR

1.5 ml reaction tubes

Molecular biology grade water

Method Overview:

5-methylcytosine (5-mC) is the predominant epigenetic mark in mammalian genomic DNA. 5-hydroxymethylcytosine (5-hmC) is a newly discovered epigenetic modification that is presumably generated by oxidation of 5-mC by the TET family of cytosine oxygenases (1,2).

Techniques exist that can identify 5-mC in genomic DNA, but the most commonly used method, bisulfite sequencing, is laborious and cannot distinguish between 5-mC from 5-hmC (3).

The EpiMark 5-hmC and 5-mC Analysis Kit can be used to analyze and quantitate 5-methylcytosine and 5-hydroxymethylcytosine within a specific locus. The kit distinguishes 5-mC from 5-hmC by the addition of glucose to the hydroxyl group of 5-hmC via an enzymatic reaction utilizing T4 β-glucosyltransferase (T4-BGT). When 5-hmC occurs in the context of CCGG, this modification converts a cleavable MspI site to a noncleavable one.

The EpiMark 5-hmC and 5-mC Analysis Kit has the following features:

- Complete conversion of 5-hmC to glucosylated 5-hmC in DNA.
- Discrimination between 5-mC and 5-hmC in CCGG sequences using enzymatic digestion and PCR amplification.
- Relative quantitation of 5-mC and 5-hmC.
- Easy-to-use protocol.

This kit contains enough material for 20 reactions. An overview of the detection procedure is summarized in Figure 1.

Outline of Procedure (see page 6 for detailed protocol)

Step I: DNA Glucosylation Reaction with T4 β-glucosyltransferase (T4-BGT)

Genomic DNA of interest is treated with T4-BGT, adding a glucose moeity to 5-hydroxymethylcytosine. This reaction is sequence-independent – therefore all 5-hmC will be glucosylated, unmodified or 5-mC containing DNA will not be affected.

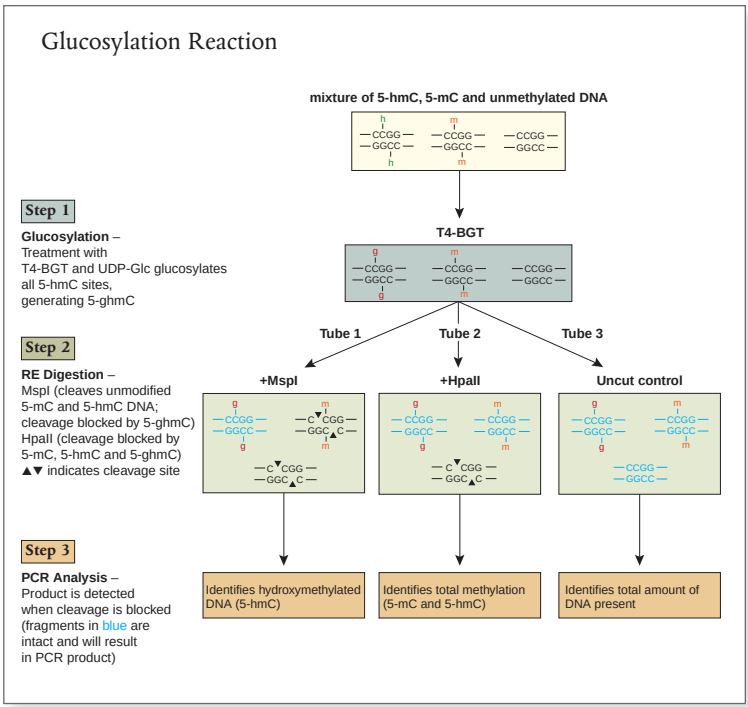
Step II: Restriction Endonuclease Digestion

MspI and HpaII recognize the same sequence (CCGG) but are sensitive to different methylation status. HpaII cleaves only a completely unmodified site: any modification (5-mC, 5-hmC or 5-ghmC) at either cytosine blocks cleavage. MspI will recognize and cleave 5-mC and 5-hmC, but not 5-ghmC.

Step III: Interrogation of the Locus by PCR

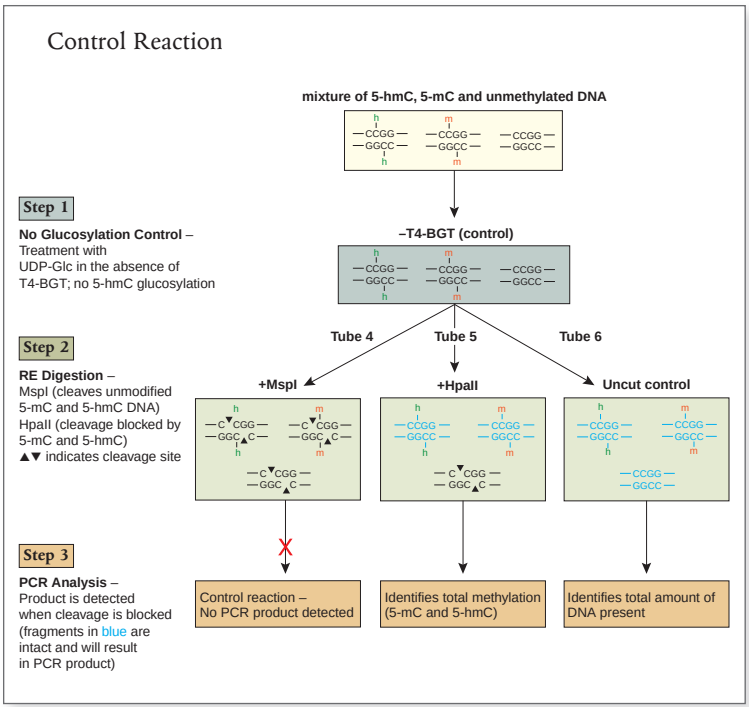
As little as 20 ng of input DNA can be used. Amplification of the experimental (glucosylated and digested) and control (mock glucosylated and digested) target DNA with primers flanking a CCGG site of interest (100–200 bp) is performed. If the CpG site contains 5-hydroxymethylcytosine, a band is detected after glucosylation and digestion, but not in the non-glucosylated control reaction (see Figure 2). Real time PCR will give an approximation of how much hydroxymethylcytosine is in this particular site.

Figure 1a: Experimental Overview



The DNA of interest is treated with T4 β-Glucosyltransferase (T4-BGT) and UDP-Glucose (UDP-Glc). T4-BGT transfers glucose from UDP-Glc onto 5-hydroxymethylcytosine (generating glucosylated 5-hydroxymethylcytosine [5-ghmC]). MspI cuts DNA containing 5-hmC, but does not cut 5-ghmC containing sites; in contrast, HpaII is blocked by any of these modifications. Presence of 5-hmC and 5-mC can be determined by PCR analysis.

Figure 1b: Experimental Overview (continued)



The DNA of interest is digested following a control reaction with UDP-Glucose (UDP-Glc) and no T4 β-Glucosyltransferase (T4-BGT), leaving 5-hmC unmodified. MspI cleaves unmodified, 5-mC and 5-hmC DNA, while HpaII cleaves only unmodified DNA.

Reaction Protocol

Step I

DNA Glucosylation and Control Reactions

1. Mix the following components in a 1.5 ml reaction tube:

REACTION COMPONENT	ADD	FINAL CONCENTRATION
Genomic DNA	5–10 µg	30 µg/ml
UDP-Glucose	12.4 µl	80 µM
NEBuffer 4	31 µl	1X
Nuclease-free water	to 310 µl	Total vol. 310 µl

2. Split the reaction mixture into two tubes (155 µl each).
3. Add 30 units (3 µl) of T4 β-glucosyltransferase (T4-BGT) to one tube. Mix well by pipetting gently up and down.

   (The second tube is the control reaction. Add 3 µl of nuclease-free water).
4. Incubate both tubes at 37°C for 12 to 18 hours.

Step II

Restriction endonuclease digestion

1. Aliquot 50 µl of each reaction mixture into three 0.2 ml PCR-strip tubes. Label tubes 1–3. Repeat for control experiment. Label tubes 4–6.
2. Add 100 units (1 µl) of MspI into tubes #1 and 4. Mix well by gently pipetting up and down.
3. Add 50 units (1 µl) of HpaII into tubes #2 and 5. Mix well by gently pipetting up and down. *(tubes #3 and 6 are controls, no restriction enzyme is added).*

DNA + T4-BGT+ UDP-Glc			DNA + UDP-Glc (Control)		
1	2	3	4	5	6
MspI	HpaII	Control (no RE)	MspI	HpaII	Control (no RE)

4. Incubate the reactions at 37°C for at least 4–16 hours.
5. Add 1 µl Proteinase K to each tube and incubate at 40°C for 30 minutes. Inactivate Proteinase K by incubating at 95°C for 10 minutes.

Step III

Analyze DNA by PCR/qPCR

End-point PCR: Protocol is provided for NEB LongAmp® Taq, which has been shown to perform well. Other PCR protocols can be substituted.

1. Add the following components to a 0.2 ml PCR reaction tube on ice:

PCR COMPONENT	50 µl PCR REACTION	FINAL CONCENTRATION
5X LongAmp *Taq* Reaction Buffer	10 µl	1X
10 mM dNTPs	1.5 µl	300 µM
10 µM Forward Primer	1 µl	0.2 µM
10 µM Reverse Primer	1 µl	0.2 µM
Template DNA (*from Step II*)	3 µl	50–100 ng
LongAmp *Taq* DNA Polymerase	1 µl	5 Units/50 µl PCR
Nuclease-free Water	To 50 µl	

2. Gently mix the reaction. If necessary, collect all liquid to the bottom of the tube by a quick spin. Overlay the sample with mineral oil if using a thermocycler without a heated lid.
3. Transfer PCR tubes from ice to a thermocycler with the block preheated to 94°C and start the cycling program.

Thermocycling conditions for a routine 3-step PCR:

CYCLE STEP	CYCLES	TEMP	TIME
Initial Denaturation	1	94°C	30 Seconds
Denaturation	30	94°C	15 Seconds
Annealing		55–65°C	30 Seconds
Extension		65°C	20 Seconds (or 50 Seconds/kb)
Final Extension	1	65°C	5 Minutes

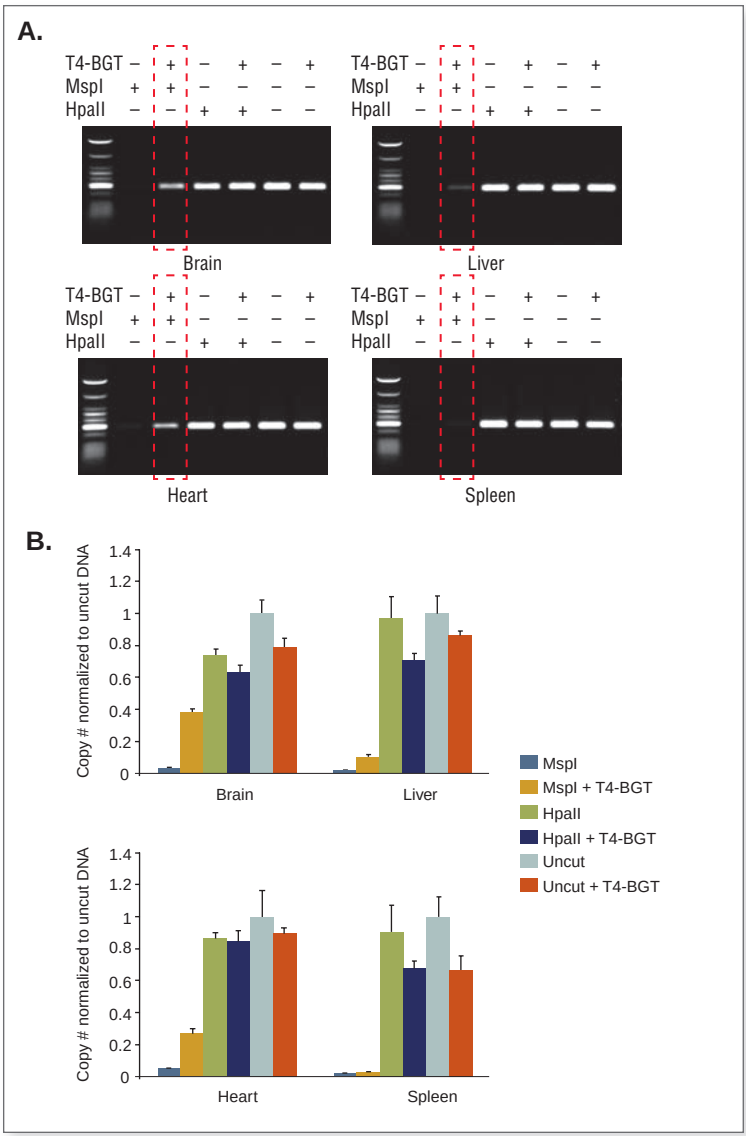
Real time PCR:
For Real Time PCR use 1–2 µl (30–60 ng) of template (from Step II) and follow the manufacturer's recommendations.

Data described in this manual was generated using the DyNAmo™ SYBR Green qPCR Kit with the Bio-Rad iQ™5 Real-time PCR Detection System.

CYCLE STEP	TEMP	TIME	CYCLES
PCR Amplification	95°C	10 min	x 1
	95°C	10 sec	x 40
	60°C	30 sec	
	72°C	20 sec	
Melting Curve	65°C and increment of 0.5°C per cycle	20 min	1

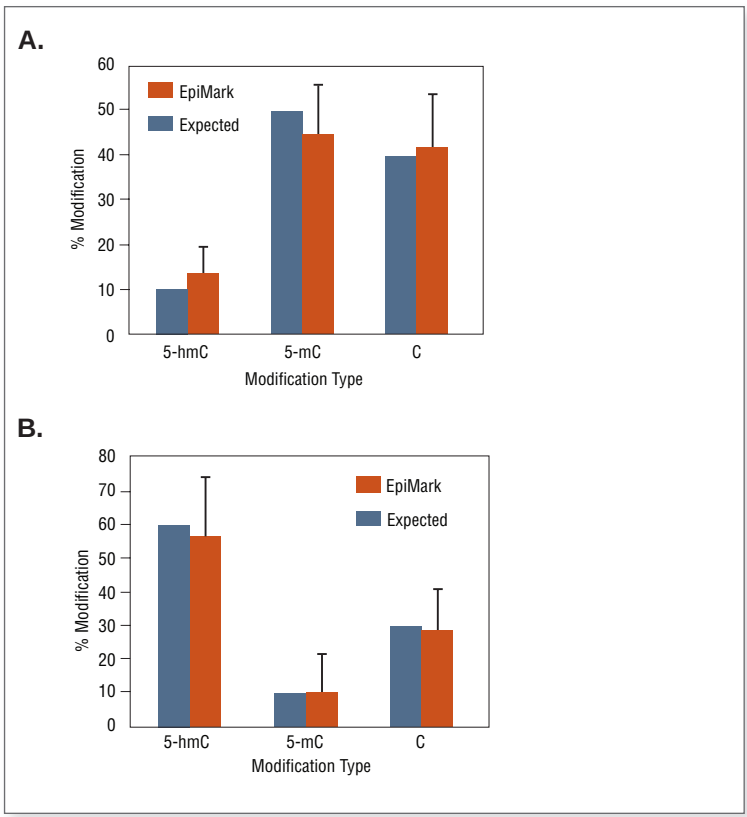
If using a standard curve to determine copy number, samples can be normalized by dividing the copy number of reactions 1–5 by the copy number of the control reaction (tube 6). If using the comparative Ct method, samples can be normalized by setting the control reaction (tube 6) as the calibrator. This normalization will give an approximate percentage of methylated (HpaII digested samples, (tubes 2 & 5) and hydroxymethylated (T4-BGT & MspI digested sample, tube 1) alleles in your sample.

Figure 2: Comparison of 5-hydroxymethylcytosine amounts at locus 12 in different mouse Balb/C tissue samples. (A), End-point PCR. (B), Real time PCR.



DNA from four mouse tissues was analyzed. For comparative purposes, real time PCR data were normalized to uncut DNA. A standard curve was used to determine copy number. The samples could be normalized by dividing the copy number of samples No 1-6 by the copy number of the control that is undigested (No 5). Boxed gel lane shows variation in 5-hmC present.

Figure 3: High sensitivity 5-hydroxymethylcytosine detection achieved by the EpiMark kit.



100 bp unmodified, 5-mC, and 5-hmC control DNAs were mixed in different ratios (blue bars), and then measured with the EpiMark hydroxymethylated DNA detection kit (orange bars). Error bars represent the standard deviation of four independent experiments. (For calculations, see Appendix)

Using the Control DNA and Primers:

Control reactions can be performed using the controls provided as input DNA to the reaction protocol.

For best results, use 1–2 ng total DNA as the input in Step I. The three types of control DNA can be mixed in any ratio desired to demonstrate the quantitative discrimination of the method.

These controls provide excellent quantitative analysis in qPCR experiments, but the number of cycles may need to be optimized to see discrimination by end-point PCR. Dilute control DNA 1:100 before using as a template for end-point PCR and amplify for approximately 25 cycles.

Frequently Asked Questions

Does the T4-BGT show any site preference?

No site preference has been observed using the reaction conditions described in the manual.

How do I know if the glucosylation of 5-hmC in my DNA is complete?

One unit of T4-BGT glucosylates 0.5 µg of T4 gt DNA to completion. Our experiments show that 10 units of T4-BGT is more than enough to glucosylate 1.5 µg of genomic DNA overnight as indicated in protocol (see page 6).

How much control DNA should I use in my qPCR?

Use 0.1 ng/µl, or dilute up to 10X in your qPCR reactions. To generate a standard curve, use 10X serial dilutions of unmodified control DNA.

Do I need to do extra purification steps for a mammalian DNA prep in order to use it in a glucosylation reaction?

No, all commercially available genomic DNA purification kits tested gave satisfactory results

What is the shelf life of the reagents supplied?

We guarantee that all reagents will remain active at least 12 months when stored at –20°C

If the quantity of genomic DNA is limited, can T4-BGT and restriction enzyme reactions be downsized to 25 or 15 µl?

Yes, while a 50 µl reaction volume is recommended, smaller reaction volumes can be used. We recommend a 50 µl reaction volume to have sufficient sample for qPCR reactions to be repeated 3 times.

Appendix

Quantitation of 5-hydroxymethylcytosine at a Specific CCGG Site:

To determine the methylation status of inner C in CCGG sites, a simple calculation can be carried out using the following formulae:

$C^{hm}CGG\% = [M_2*(C_1/C_2) - M_1]/C_1$;
$C^{m}CGG\% = [H_1 - M_2*(C_1/C_2)]/C_1$;
$CCGG\% = (C_1 - H_1)/C_1$;

in these calculations, the parameters are:

M_2: qPCR value in the sample of genomic DNA with MspI and T4-BGT (tube 1)
H_2: qPCR value in the sample of genomic DNA with HpaII and T4-BGT (tube 2)
C_2: qPCR value in the sample of genomic DNA with T4-BGT only (tube 3)
M_1: qPCR value* in the sample of genomic DNA with MspI (tube 4)
H_1: qPCR value in the sample of genomic DNA with HpaII (tube 5)
C_1: qPCR value in the sample of genomic DNA only (tube 6)
*: qPCR value can be raw Ct values, or normalized Ct values.

Sample Calculation:

Single dataset (of 9) used to generate Figure 3b

M1 (tube 4)	0.10
C1 (tube 6)	1.91
M2 (tube 1)	0.69
C2 (tube 3)	1.00
H1 (tube 5)	1.49
H2 (tube 2)	0.60

hmC = [0.69 x 1.91/1.00 – 0.1]/1.91 = 64%

mC = [1.49 – 0.69 x 1.91/1.00]/1.91 = 9%

C = [1.91 – 1.49]/1.91 = 22%

References

1. Kriaucionis, S. and Heintz, N. (2009) *Science* 324, 929–930. Epub 2009 Apr 16.
2. Tahiliani, M., Koh, K.P., Shen, Y., Pastor, W.A., Bandukwala, H., Brudno, Y., Agarwal, S., Lyer, L.M., Liu, D.R., Aravind, L., Rao, A. (2009) *Science* 324, 930–935. Epub 2009 Apr 16.
3. Huang, Y, Pastor, W.A., Shen, Y., Tahiliani, M., Liu, D.R., Rao, A. (2010) *PLoS One*. Epub 2010 Jan 26;5(1):e8888.

Ordering Information

PRODUCT	NEB #	SIZE
EpiMark 5-hmC and 5-mC Analysis Kit	E3317S	20 reactions
COMPANION PRODUCTS		
T4 Phage β-glucosyltransferase (T4-BGT)	M0357S/L	500/2,500 units
MspI	R0106S/L	5,000/25,000 units
MspI, high concentration	R0106T/M	5,000/25,000 units
HpaII	R0171S/L	2,000/10,000 units
HpaII, high concentration	R0171M	10,000

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