Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific EpiJET 5-hmC and 5-mC Analysis Kit

#K1501 **25 rxns**

Lot __ **Expiry Date __**

Store at -20 ºC

www.thermoscientific.com/onebio

KIT COMPONENTS

Component	#K1501 25 rxns
T4 β-glucosyltransferase, 5 U/µL	25 µL
10X Epi Buffer	1.2 mL
10x UDP-glucose	500 µL
Epi MspI	50 µL
Epi HpaII	25 µL
5-hmC Control DNA, 25 nM *	20 µL
5-mC Control DNA, 25 nM *	20 µL
Unmodified Control DNA, 25 nM *	20 µL
Control primer 1, 10 µM **	20 µL
Control primer 2, 10 µM **	20 µL
Water, nuclease-free	1.25 mL

* Each Control DNA consists of a double-stranded DNA fragment containing a single Epi MspI/HpaII recognition site (CCGG) with different modification of the internal C (see the Control DNA sequence in the Appendix section).

** Primer sequences can be found in the Appendix section.

Rev.1

DESCRIPTION

The EpiJET™ 5-hmC and 5-mC Analysis Kit provides a total assessment of both cytosine modifications - 5-hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC) - within specific DNA loci.

The EpiJET 5-hmC and 5-mC Analysis Kit includes three advanced enzymes: T4 BGT, Epi MspI and Epi HpaII. The kit takes advantage of the T4 phage β-glucosyltransferase (T4 BGT) ability to specifically modify 5-hmC residues by adding glucose moiety to 5-hmC and the sensitivity of restriction enzyme Epi MspI to glucosylated 5-hmC. The T4 BGT is formulated for highly specific, complete and fast glucosylation of 5-hmC in 15 min.

The Epi MspI and Epi HpaII are isoschisomers with differing sensitivity to internal C methylation of CCGG site. Epi MspI completely digests genomic DNA in 4 hours when cytosine, 5-methylcytosine, or 5-hydroxymethylcytosine is within its recognition sequence, but its activity is completely blocked when 5-hmC is glucosylated. Epi HpaII cleaves unmodified CCGG site only.

The analysis involves DNA sample glucosylation and subsequent Epi MspI and Epi HpaII digestion. Restriction digestion products are then analyzed by qPCR with a primer pair flanking CCGG site of interest.

ADDITIONAL MATERIALS AND EQUIPMENT REQUIRED

- Pipettes and pipette tips.
- Sterile tubes for qPCR and enzymatic reactions.
- Mini-Centrifuge/Vortex.
- Disposable gloves.
- Heat block or waterbath capable of heating up to 37 °C and 80 °C.
- Locus specific primers.
- qPCR reagents.
- qPCR instrument.

IMPORTANT NOTES

- Mix DNA glucosylation and Epi MspI/HpaII digestion reaction components at room temperature.
- To minimize the possibility of pipetting errors during the DNA glucosylation step, prepare enough reaction master mix (including DNA sample, but not including T4 BGT) for four reactions plus 10% extra. Mix thoroughly and dispense the mixture into four sterile tubes (49 µL each). Add 1 µL of nuclease-free water into tubes **1**, **2** and **4**, add 1 µL of T4 BGT into tube **3** and follow protocol recommendations.
- The T4 BGT should always be the last component added to the reaction mixture.
- DNA purity is an important factor for successful digestion by Epi MspI/HpaII. We recommend use of spin column-based kits such as the Thermo Scientific™ GeneJET™ Genomic DNA Purification Kit (Cat #K0721/2) for genomic DNA purification.
- When the external C in the CCGG sequence is modified, cleavage with Epi MspI/HpaII is blocked.

Guidelines for primer design

For effective analysis of 5-mC, 5-hmC and C by qPCR, locus-specific primers that flank the CCGG site of interest must be used. To design appropriate primers, use primer design software or follow the general recommendations outlined below:

- Primer GC content: 30-60 %.
- Primer length: 18-30 nucleotides.
- Optimal primer melting temperature (Tm): 60 °C. Differences in Tm of the two primers should not exceed 2°C.
- Optimal amplicon length: 70-150 bp.
- Avoid more than two G or C nucleotides in last five nucleotides at the 3'-end of the primer to lower the risk of nonspecific priming.
- Avoid designing primers around secondary structures in the amplicon.
- Avoid primer self-complementarities, complementarities between the primers and direct repeats in a primer to prevent hairpin formation and primer dimerization.
- When designing a new assay, it is recommended to verify the PCR product specificity by gel electrophoresis, as melting temperatures of a specific product and primer-dimers may overlap depending on the sequence composition.

Guidelines for qPCR

- During qPCR setup, it is important to avoid DNA cross-contamination. We recommend using a dedicated set of pipettes for qPCR to minimize contamination.
- The accuracy of qPCR is highly dependent on accurate pipetting and thorough mixing of individual reaction components. Take extra care to avoid pipetting errors during qPCR setup and when preparing templates for qPCR. Use of 2-3 technical replicates is highly recommended.
- For calculation of C modification percentages use the formulas provided in the Calculation of 5-hmC, 5-mC and C percentages section. To determine the PCR efficiency value prepare a standard calibration curve by diluting the “Control (untreated)” sample.

Calculation of 5-hmC, 5-mC and C percentages

% of 5-hmC = $100 / (1 + E)^{Cq3-Cq1}$

Where:

Cq1 is the threshold cycle of “Control (untreated)” sample.

Cq3 is the threshold cycle of “+T4 BGT+Epi MspI” sample.

E is the PCR efficiency value.

% of 5-mC = $100 / (1 + E)^{Cq2-Cq1} - 100 / (1 + E)^{Cq3-Cq1}$

Where:

Cq1 is the threshold cycle of “Control (untreated)” sample.

Cq2 is the threshold cycle of “+Epi HpaII” sample.

Cq3 is the threshold cycle of “+T4 BGT+Epi MspI” sample.

E is the PCR efficiency value.

% of C = $100 - 100 / (1 + E)^{Cq2-Cq1}$

Where:

Cq1 is the threshold cycle of “Control (untreated)” sample.

Cq2 is the threshold cycle of “+Epi HpaII” sample.

E is the PCR efficiency value.

For reliable results, data from qPCR reactions with amplification effcencies from 0.9 to 1.1 should be used (1 corresponds to 100% efficiency).

PROTOCOL

A. DNA Glucosylation

- Read the Important Notes section before starting.
- Gently vortex and briefly centrifuge the 10X Epi Buffer and 10X UDP-glucose solution after thawing.

1. Mix the following components in sterile tubes at room temperature:

Component	Control (untreated)	+ Epi HpaII	+ T4 BGT + Epi MspI	+ Epi MspI
	1	2	3	4
Genomic DNA	0.05 – 1 µg	0.05 – 1 µg	0.05 – 1 µg	0.05 – 1 µg
10X UDP-glucose	5 µL	5 µL	5 µL	5 µL
10X Epi Buffer	5 µL	5 µL	5 µL	5 µL
T4 β-glucosyltransferase, 5 U/µL	-	-	1 µL	-
Water, nuclease-free	to 50 µL	to 50 µL	to 50 µL	to 50 µL
Total volume	50 µL	50 µL	50 µL	50 µL

2. Gently mix (do not vortex) and spin briefly.
3. Incubate the reactions at 37 °C for 15 minutes.

B. Epi HpaII and MspI digestion

1. Add 1 µL of Epi Hpa II into tube **2**.
2. Add 1 µL of Epi MspI into tubes **3** and **4**.
3. Add 1 µL of nuclease-free water into tube **1.**
4. Gently mix (do not vortex) and spin briefly to collect drops.
5. Incubate the reactions at 37 °C for 4 hours.
6. Stop the reaction by heating at 80 °C for 20 minutes.

C. qPCR amplification

Use primers that flank the CCGG site of interest.

Use 1-2 µL (1-40 ng) of template prepared in Step B and follow the manufacturer’s recommendations for qPCR reaction setup and thermal cycling conditions.

Recommendations for qPCR

- The parameters below are recommended for qPCR using Thermo Scientific™ Maxima™ SYBR Green/ROX qPCR Master Mix (Cat #K0221/2/3).
- To minimize the possibility of pipetting errors, prepare a reaction master mix by adding the following components (excluding template DNA) for each 20 µL reaction to a tube at room temperature:

Component	Volume
Maxima SYBR Green/ROX qPCR Master Mix (2X)	10 µL
Forward Primer	0.3 µM
Reverse Primer	0.3 µM
Template (genomic DNA from Step B)	1-2 µL (1-40 ng)
Water, nuclease-free	to 20 µL
Total volume	20 µL

- Mix the reaction master mix thoroughly and dispense the desired volume into PCR tubes or plates.

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- Add template DNA [1-2 µL (1-40 ng)] of genomic DNA prepared in Step B) to the individual PCR tubes or wells containing master mix. Centrifuge briefly if needed.

Note. *To minimize inaccuracies associated with pipetting smaller volumes we recommend to dilute template DNA 3-fold and use 3-6 µL.*

- Program the thermal cycler according to the recommendations below:

Step	Temperature, °C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60*	30 s	
Extension	72	30 s	

* use the optimal annealing temperature for your primer pair.

- Use two-step cycling protocol, if the optimal annealing temperature for your primer pair is 60 °C:

Step	Temperature, °C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing/ Extension	60	60 s	

Note. *Melting curve analysis may be performed to verify the specificity of the PCR product. Primer-dimers may occur during PCR if the primer design is not optimal. The dimers are distinguished from the specific product through their lower melting point.*

Interpretation of results

Result
If Cq3 ≈ Cq4, Cq1 ≈ Cq2 and (Cq3 ≈ Cq4) – (Cq1 ≈ Cq2) ≥ 4.5, **Full methylation.** Methylation blocked the cleavage by Epi HpaII. CCGG site is fully 5-methylated.
If Cq1 ≈ Cq2 ≈ Cq3 and Cq4 - (Cq1 ≈ Cq2 ≈ Cq3) ≥ 4.5, **Full hydroxymethylation.** Glucosylation blocked cleavage by Epi MspI. CCGG site is fully 5-hydroxymethylated.
If Cq2 ≈ Cq3 ≈ Cq4 and (Cq2 ≈ Cq3 ≈ Cq4) – Cq1 ≥ 4.5, **No cytosine modification**. CCGG site does not contain 5-hmC or 5-mC bases. Epi HpaII and Epi MspI are able to cleave CCGG.
If Cq1 < Cq2 < Cq3 < Cq4, **Partial cytosine modification.** Cleavage levels represent modification level in the CCGG site of interest. Calculate into 5-hmC, 5-mC and C percentage.
If Cq1≈ Cq2 ≈ Cq3 ≈ Cq4*, **No cleavage.** No Epi MspI/HpaII recognition site or CCGG sequence is modified at the external C (Epi MspI/HpaII cleavage is blocked).

Cq1 - threshold cycle of "Control (untreated)" sample
Cq2 - threshold cycle of "+Epi HpaII" sample
Cq3 - threshold cycle of "+T4 BGT+Epi MspI" sample
Cq4 - threshold cycle of "+Epi MspI" sample
* ≈ stands for ∆Cq ≤ 0.5

Note. *Modification of external C in the **C**CGG sequence blocks the activity of Epi MspI/HpaII. Levels of external C methylation vary depending on sample type and origin.*

CONTROL REACTIONS

A. Control DNA glucosylation:

- Read the Important Notes section before starting.
- Gently vortex and briefly centrifuge 10X Epi Buffer and 10X UDP-glucose solution after thawing.

1. Mix the following components in sterile tubes at room temperature:

Component	Control (untreated)	+Epi HpaII	+ T4 BGT + Epi MspI	+Epi MspI
	1	**2**	**3**	**4**
Control DNA, 25nM *	1 µL	1 µL	1 µL	1 µL
10X UDP-glucose	5 µL	5 µL	5 µL	5 µL
10X Epi Buffer	5 µL	5 µL	5 µL	5 µL
T4 β – glucosyltransferase, 5 U/µL	-	-	1 µL	-
Water, nuclease-free	to 50 µL	to 50 µL	to 50 µL	to 50 µL
Total volume	50 µL	50 µL	50 µL	50 µL

* Choose respective type of Control DNA upon your needs. It is also possible to prepare a mixture of three types of Control DNA at any ratio to show the quantitative discrimination of the method.

2. Gently mix (do not vortex) and spin briefly.
3. Incubate the reactions at 37 °C for 15 minutes.

B. Epi HpaII and Epi MspI digestion

1. Add 1 µL of Epi HpaII into tube **2**.
2. Add 1 µL of Epi MspI into tubes **3** and **4**.
3. Add 1 µL of nuclease-free water into tube **1**.
4. Gently mix (do not vortex) and spin briefly.
5. Incubate all tubes at 37 °C for 4 hours.
6. Stop the reaction by heating at 80 °C for 20 minutes.

For qPCR (Step C), dilute the Control DNA 1000 times.

C. qPCR amplification

- The parameters below are suggested for qPCR using Maxima SYBR Green/ROX qPCR Master Mix (Cat #K0221/2/3).
- To minimize the possibility of pipetting errors, prepare a reaction master mix by adding the following components (excluding template DNA) for each 20 µL reaction to a tube at room temperature:

Component	Volume
Maxima SYBR Green/ROX qPCR Master Mix (2X)	10 µL
Control primer 1, 10 µM	0.6 µL
Control primer 2, 10 µM	0.6 µL
Control DNA (from **Step B)**	5 µL *
Water, nuclease-free	3.8 µL
Total volume	20 µL

* For qPCR use 5 µL of diluted Control DNA (from Step B).

- Mix the reaction master mix thoroughly and dispense the desired volume into PCR tubes or plates.
- Add template DNA (5 µL of diluted Control DNA prepared in Step B) to the individual PCR tubes or wells containing master mix.
- Centrifuge briefly if needed.
- Program the thermal cycler according to the recommendations below:

Step	Temperature, °C	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing/ Extension	60	60 s	

Expected qPCR results:

Using 5-hmC Control DNA
1) ΔCq value between "Control (untreated)", "+Epi HpaII" and "+T4 BGT+MspI" samples should be ≤ 0.5 cycle,
2) ΔCq value between "+MspI" and "+T4 BGT+MspI" samples should be ≥ 4.5 cycles.

Using 5-mC Control DNA
1) ΔCq value between "Control (untreated)" and "+Epi HpaII" samples should be ≤ 0.5 cycle,
2) ΔCq value between "+T4 BGT+Epi MspI" and "+Epi MspI" samples should be ≤ 0.5 cycle,
3) ΔCq value between "+Epi MspI" and "+Epi HpaII" samples should be ≥ 4.5 cycles.

Using unmodified Control DNA
1) ΔCq value between "+Epi HpaII"/ "+Epi MspI" and "Control (untreated)" samples should be ≥ 4.5 cycles.

APPENDIX

Control DNA amplicon sequence:

5'-
CTGTCATGGTGACAAAGGCATC**C***GGCAGAAATGCCCACACAGCCTCTTTAAC
CAGCACGC
CAACCGCCTCTGCTTCGGCCCTGGTCACGCAGCTGACAAGGTCTTCATAATAG
AGAAATC
CTG– 3'

C* – cytosine of different modifications.

Primer complementarity regions underlined.

Sequences of control primers:
Control primer 1: 5'- CTGTCATGGTGACAAAGGCATC -3'
Control primer 2: 5'-CAGGATTTCTCTATTATGAAGACCTTG-3'

TROUBLESHOOTING

Problem	Cause and Solution
Incomplete digestion/no digestion of non-glucosylated gDNA	**Low gDNA purity.** • For high quality genomic DNA purification use commercially available gDNA purification kits, such as GeneJET Genomic DNA Purification Kit (Cat #K0721/2). **Sample contains modified external cytosine in the CCGG sequence.**
Unexpected control reaction results	**Improper reaction setup.** • Prepare DNA glucosylation and Epi MspI/HpaII digestion reactions at room temperature. • The T4 β- glucosyltransferase enzyme should always be the last component added to the reaction mixture. **Pipetting errors.** • The accuracy of qPCR is highly dependent on accurate pipetting and thorough mixing of individual reaction components. Take extra care to avoid pipetting errors during qPCR setup and when preparing templates for qPCR.
qPCR irregularities	**Too high volume of sample was used for qPCR.** • If template DNA is used for qPCR directly after Epi MspI/HpaII restriction digest, do not use more than 1-2 µL of restriction solution for 20 µL of reaction mix, or use diluted samples. • For some genomic DNA samples, more reliable results can be achieved using less template (2-4 ng instead of 20-40 ng for one 20 µL reaction).

PRODUCT USE LIMITATION
This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.
Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.
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