Analysis of 5-methylcytosine and 5-hydroxymethylcytosine Levels in Human Brain and Liver DNA Samples using the EpiMark™ 5-hmC and 5-mC Analysis Kit

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Introduction

In the mammalian genome, enzymatic DNA methylation is the predominant epigenetic modification. DNA methylation is dynamic, heritable, and reversible, providing important determinants for an array of epigenetic states regulating phenotype and gene expression patterns (1). Cytosine residues undergo modification at the carbon-5 position by DNA (cytosine-5) methyltransferases (2) to form 5-methylcytosine (5-mC). In the genome, variable percentages of 5-mC are enzymatically oxidized to 5-hydroxymethylcytosine (5-hmC) by a family of Ten Eleven Translocation (TET1/2/3) enzymes. 5-mC is predominately found in CpG dinucleotide sequences, although small percentages are also found in non-CpG (CpHpG and CpHpH) context (3). The functional role of CpG methylation in mammalian genome is structural, possibly involving disruption of transcription factor binding and/or recruitment of transcriptional repressors such as methyl CpG binding proteins (MeCPs). Compared to 5-mC, 5-hmC may influence chromatin structure and local transcriptional activity by repelling 5-mC binding proteins or recruiting 5-hmC specific proteins. Indeed, in a previous report it was demonstrated that methyl-binding protein MeCP2 does not recognize or bind to 5-hmC (4). More recent reports using several other methyl binding proteins, including MBD1, MBD2, and MBD4, support this hypothesis (5). Because 5-hmC is present in the mammalian genome, specifically in gene bodies (intragenic regions) and enriched at promoters, but largely absent from non-gene regions in DNA, suggests its biological functions differ from 5-mC (6). The presence of 5-hmC in gene bodies is positively correlated with gene expression levels. Therefore, the importance of studying 5-mC and 5-hmC separately for their biological roles is of scientific interest.

The EpiMark 5-hmC and 5-mC Analysis Kit offers a robust method for the quantitative determination of 5-hmC in CpG context, embedded in CCGG sites in the mammalian genome. This enzymatic approach utilizes MspI sensitivity to glucosylated 5-hmC in a simple three step protocol (Figure 1).

It is known that certain areas of human brain and liver DNA have densely methylated CpG sites (7). Understanding the amount of 5-mC and 5-hmC percentage in specific tissues will help to elucidate the role of 5-hmC in epigenetic regulation. In this application note, a CpG site from four different genes (EGFR, PRKAA2, RPL11P5, and VANGL1) was interrogated using the Epimark Kit in both liver and brain tissue DNA, in order to determine the percentage of 5-mC and 5-hmC at specific loci.

Materials

- Genomic DNA
- EpiMark™ 5-hmC and 5-mC Analysis Kit and qPCR reagents
- qPCR primers

Protocol

1. Assemble glucosylation reaction on ice in nuclease free, 0.5 ml microcentrifuge tubes (x, X, y, and Y are dependent on DNA concentration):

TUBE	CONTROL	EXPERIMENTAL
H_2O	X µl	Y µl
10X NEBuffer 4	15 µl	10 µl
gDNA	x (4.5 µg)	y (3 µg)
EpiMark BGT	0 µl	1 µl
EpiMark UDP-glc	1.5 µl	1 µl
Total	150 µl	100 µl

Mix the reaction gently by tapping or pipetting. Briefly centrifuge the reaction mixture and incubate at 37°C for 12 to 18 hrs.

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2. Assemble restriction enzyme digestion reactions on ice in nuclease-free, 0.5 ml microcentrifuge tubes:

TUBE	1	2	3	4	5
H_2O	–	–	–	–	1 µl
Mix from tube C	50 µl	50 µl	–	–	50 µl
Mix from tube E	–	–	50 µl	50 µl	–
EpiMark MspI (100U/µl)	1 µl	–	1 µl	–	–
EpiMark HpaII (50U/µl)	–	1 µl	–	1 µl	–
Total	51 µl	51 µl	51 µl	51 µl	51 µl

Mix the reaction gently by tapping or pipetting. Briefly centrifuge the reaction mixture and incubate at 37˚C for 1 hr. Digested samples can be diluted up to 2-fold for use with PCR.

3. Perform qPCR in triplicate (volumes are for a single 10 µl reaction):

TUBE	1	2	3	4	5
H_2O	3.2 µl	3.2 µl	3.2 µl	3.2 µl	3.2 µl
2X SYBR Green MM	5 µl	5 µl	5 µl	5 µl	5 µl
Primer 1 (400 nM final conc.)	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl
Primer 2 (400 nM final conc.)	0.4 µl	0.4 µl	0.4 µl	0.4 µl	0.4 µl
Sample	1 µl	1 µl	1 µl	1 µl	1 µl
Total	10 µl	10 µl	10 µl	10 µl	10 µl

Fig 1: EpiMark 5-hmC and 5-mC Analysis Kit Workflow

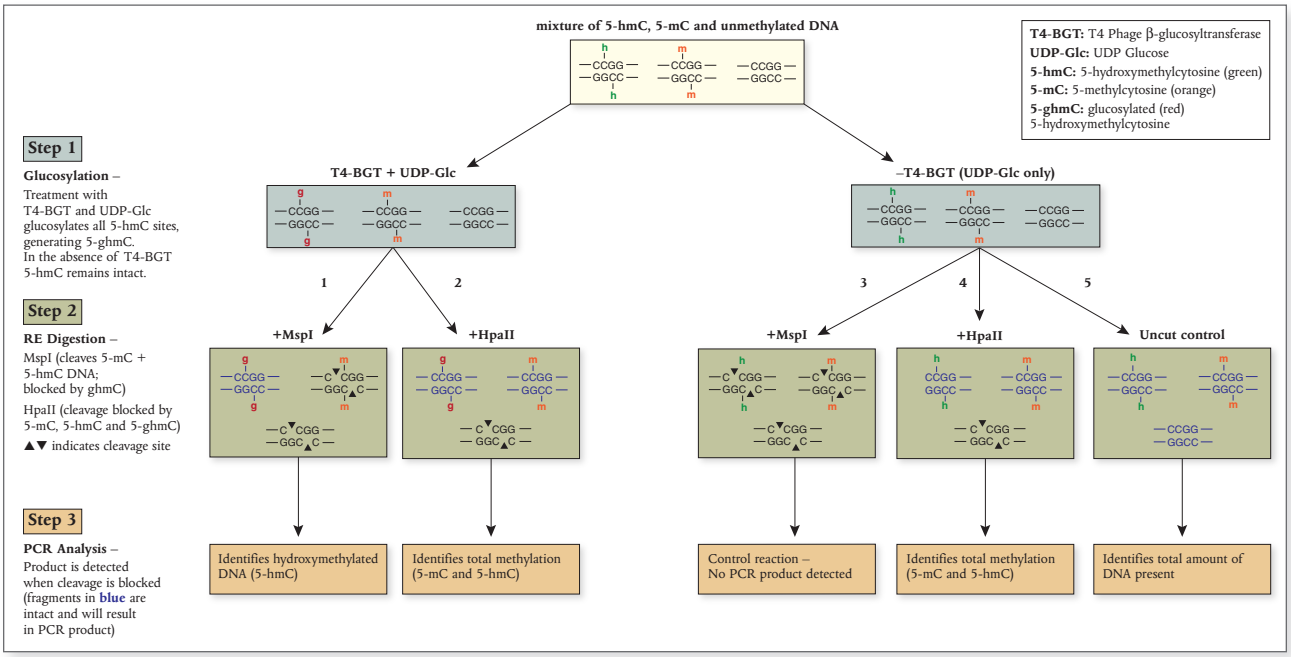

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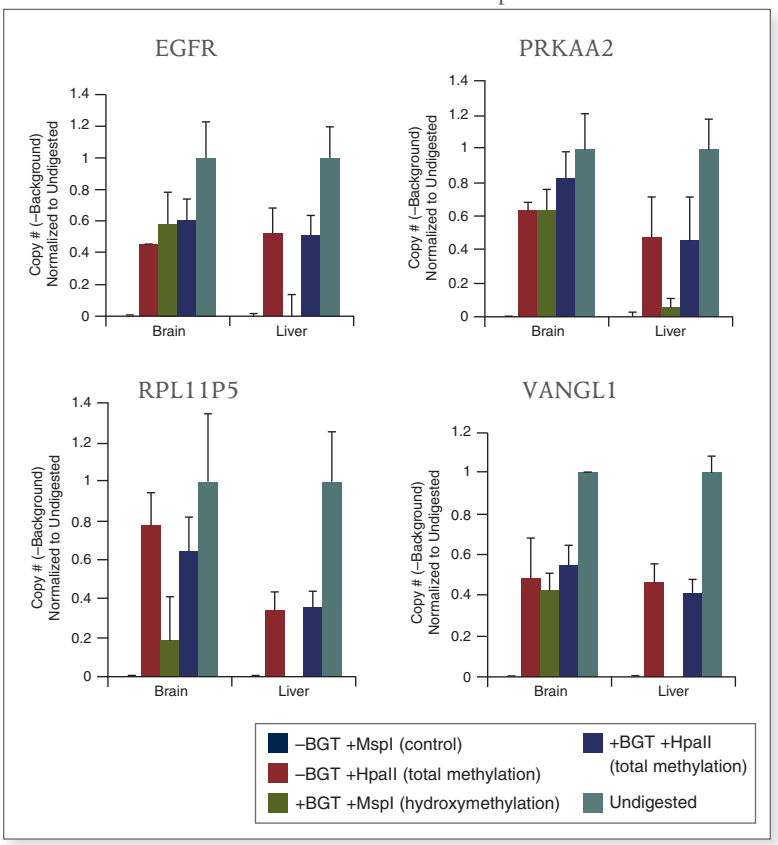
Fig 2: Analysis of specific genes in human brain and liver samples shows a variation in the amounts of 5-hmC and 5-mC present.



Reactions were set up according to protocol on page 3 and analyzed by qPCR.

Results

The densely methylated CpG sites (EGFR, PRKAA2, RPL11P5 and VANGL [all >60% methylated]) were chosen after bisulphite DNA sequencing of human brain DNA. Quantitative PCR analysis of 5-mC and 5-hmC were consistent with the results obtained with bisulphite sequencing (data not shown). The results showed that total methylation was similar for the genes examined in both brain and liver tissues. However, there were variations in the amount of 5-hmC present. Specifically, brain DNA was consistently hydroxymethylated from 20-60% at each interrogated locus, whereas liver DNA contained almost no 5-hmC at the same sites.

Summary

The EpiMark 5-hmC and 5-mC Analysis Kit was used to interrogate and quantify 5-mC and 5-hmC in human brain and liver DNA at four different genetic loci. Similar to 5-mC, 5-hmC also displays tissue specific patterns that may be involved in regulating gene expression in different tissues. After glucosylation and restriction enzyme digestion, the sample DNAs were used for qPCR with gene specific primers. Direct measurement of the percent cytosine, 5-mC and 5-hmC was possible with small quantities of input DNA. These tools can be utilized with any genomic DNA that contains 5-mC or 5-hmC modified bases for both detection and quantification, when coupled with qPCR. Furthermore, one could utilize standard PCR and gel analysis of PCR products to determine the presence or absence of 5-mC or 5-hmC at CCGG loci.

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