A New, Highly-Specific Monoclonal Antibody and Kits to Study DNA Hydroxy-Methylation in the Genome

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Abstract

5-hydroxymethyl-cytosine (hmC)

There is substantial interest and speculation in the role of the "sixth DNA base," 5-hydroxymethylcytosine (5-hmC), although its precise function has not yet been elucidated. Since its discovery in neuronal Purkinje, granule and ES cells, studies of this new modified DNA base have been limited by the lack of high-quality, validated tools and technologies that discriminate hydroxy-methylation from methylation in regulating genome expression. Obtaining a specific assay for 5-hmC is particularly important since standard bisulfite sequencing cannot distinguish between these two types of methylation.

Here we present new, highly-specific monoclonal antibodies and kits for the differential study of the functions of 5-hmC, 5-mC, and unmodified C, with new data on Diagenode's new HMC antibody, "hMeDIP" and MeDIP kits. Our monoclonal antibody for 5-hmC (Diagenode's "HMC" antibody; Cat. No. MAb-633HMC-050 and MAb-633HMC-100) has been extensively validated using dotblots, immunofluorescence, hMeDIP, as well as hMeDIP followed by next-gen sequencing. Our new hMeDIP kit includes the new HMC antibody, and positive (ie, fully hydroxy-methylated) and negative (ie, both unmethylated and fully-methylated) controls. We expect that these kits and reagents may represent the first and possibly most compelling set of validated products available for the study of 5-hmC and 5-mC.

We provide data showing the specificity of 5-hmC vs. both 5-mC and unmodified C using dot blots, Dual MeDIP and hMeDIP. In situ cellular staining on interphase ES cells (data not shown) indicates distinct 5-hmC staining, both in overall brightness and in binding patterns, from that obtained with the Diagenode 5-mC antibody (Cat . No. MAb-335MEC-100 and MAb-335MEC-500).

Our data are consistent with distinct roles of 5-hmC and 5-mC; our new kits and antibody open the door to novel epigenetics studies, and to clarifying the role of 5-hmC in differentiation, displacing methyl-binding proteins, regulating DNA repair, recruiting chromatin modifiers, and other important functions.

Introduction

5-hydroxymethylcytosine's (5-hmC) has been recently discovered in mammalian DNA by two US groups (Kriaucionis & Heintz, Science, 2009 and Tahiliani et al., Science, 2009). This results from the enzymatic conversion of 5-methyl-cytosine into 5-hydroxymethylcytosine by the TET family of oxygenases. So far, the 5-hmC bases have been identified in Purkinje neurons, in granule cells and embryonic stem cells where they are present at high levels (up to 0,6% of total nucleotides in Purkinje cells).

Preliminary results indicate that 5-hmC may have important roles distinct from 5-mC. Although its precise role has still to be shown, early evidence suggests a few putative mechanisms that could have big implications in epigenetics: 5-hydroxymethylcytosine may well represent a new pathway to demethylate DNA involving a repair mechanism converting hmC to C and, as such open up entirely new perspectives in epigenetic studies.

Due to the structural similarity between 5-mC and 5-hmC, these bases are experimentally almost indistinguishable. Recent articles demonstrated that the most common approaches (eg. enzymatic approaches, bisulfite sequencing) do not account for hmC. The development of the affinity-based technologies appears to be the most powerful way to differentially and specifically enrich 5mC and 5hmC sequences. The results shown here illustrate the use of a unique monoclonal antibody against hmC that has been fully validated in various technologies.

Methods

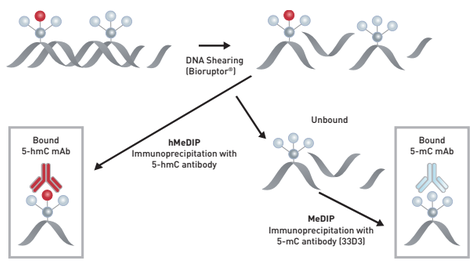


Figure 1. Principle of the Dual MeDIP assay

Dual MeDIP is a 2-step immunocapture approach developed by Diagenode to selectively enrich hydroxymethylated or methylated DNA. Genomic DNA is randomly sheared by sonication, then immunoprecipitated sequentially – first with an antibody that specifically recognizes 5-hmC, and then with an antibody against 5-mC in the unbound fraction of the first IP assay. This protocol confirms the efficiency and specificity of Diagenode's antibodies against 5-hmC and 5-mC using PCR controls.

Results

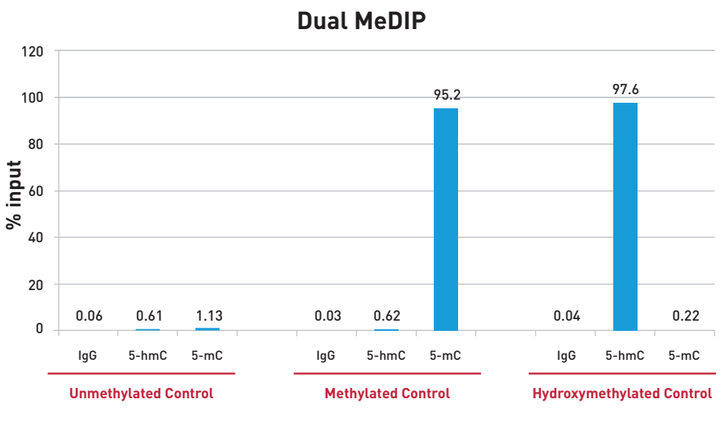


Figure 2. Dual DNA IP results obtained with Diagenode's hMeDIP kit and antibodies against methylated and hydroxymethylated C.

A methylated DNA IP (MagMeDIP) was performed on the unbound fraction of the hydroxymethylated DNA IP (hMeDIP) assays using the Diagenode antibody directed against 5-hydroxymethylcytosine, 5-methylcytosine and the "5-hmC, 5-mC & 5-C DNA standard pack" (Cat. No. AF-101-0002). IgG isotype antibody from rat (Cat. No. AF-105-0025) was used as a negative control. The DNA was prepared with the GenDNA module of the hMeDIP kit and sonicated with our Bioruptor® (UCD-200/300 series) to have DNA fragments of 300-500 bp. 1 µg of mouse ES E14 cells DNA were spiked with 0.025 ng of each PCR methylation control. The IP'd material has been analysed by qPCR using the primer pair specific for the control sequence.

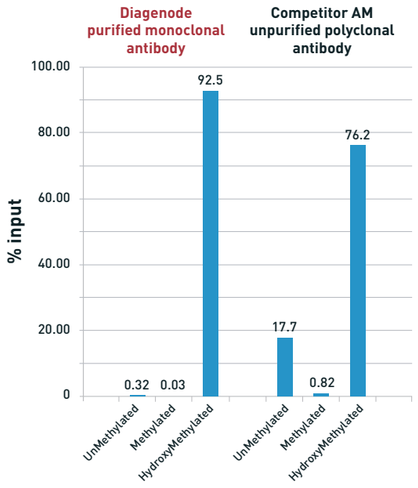
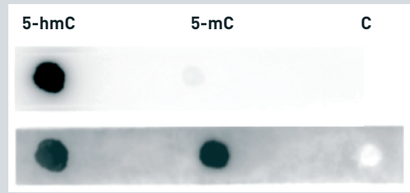


Figure 3. Comparison between Diagenode rat purified monoclonal antibody and competitor AM rabbit unpurified polyclonal antibody against 5-hmC using Diagenode's hMeDIP kit.

Hydroxymethylated DNA IP (hMeDIP) was performed using two antibodies directed against 5-hydroxymethylcytosine: 1) Diagenode's monoclonal antibody (Cat. No. MAb-633HMC-100), 2) Competitor AM's polyclonal antibody. The IgG isotype antibodies from rat (Cat. No. AF-105-0025) and rabbit (Cat. No. AF-105-0025) were used as a negative controls (data not shown). 1 µg of Hela cells DNA was prepared and sonicated with our Bioruptor® (UCD-200/300 series) to obtain DNA fragments of 300-500 bp. Unmethylated, methylated and hydroxymethylated spike-in controls (Cat. No. AF-101-0002) have been used and the hMeDIP has been carried out following the procedure of the hMeDIP kit (Cat. No. AF-104-0016). Finally qPCR using specific primer pairs for the unmethylated, methylated and hydroxymethylated DNA sequences has been performed.

Specificity of our 5-hmC and 5-mC antibodies by dot blot



A. First probing, with 5-hmC antibody

B. Second probing with 5-mC antibody (no wash between probings)

Figure 4. 200 ng of each PCR product were spotted on the membrane. Panel A: the membrane was first incubated with 4 µg/ml 5-hmC antibody (Cat. No. MAb-633HMC-100) (dilution 1:500). Panel B: incubation of the same membrane with the 5-mC antibody (Cat. No. MAb-081-100) (dilution 1 :250). Note that the membrane hasn't been stripped after the 5-hmC incubation. The left spot represents the 5-hmC signal. This result confirms that equal amounts of hmC and mC bases have been spotted on the membrane.

Efficacy of Diagnode's hMeDIP assay on both single- and double-stranded DNA

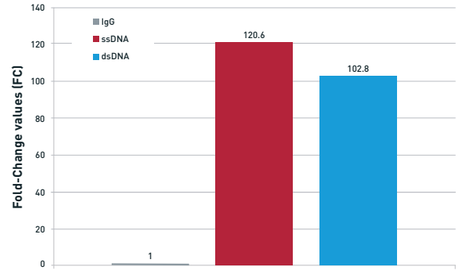


Figure 5. A hydroxymethylated DNA IP (hMeDIP kit; Cat. No. AF-104-0016) was performed using the Diagenode antibody directed against 5-hydroxymethylcytosine (Cat. No. MAb-633HMC-100). The IgG isotype antibody from rat (Cat. No. AF-105-0025) was used as a negative control. 1 µg of E14 ES cells was prepared with the GenDNA module of the hMeDIP kit and sonicated with our Bioruptor® (UCD-200/300 series) to obtain DNA fragments of 300-500 bp. The IP'd material has been analysed by qPCR using the primer pair specific for the Sfi1 (splicing factor 1) gene.

Ordering information

Description	Cat. No.	Format
5-hmC monoclonal antibody (rat)	MAb-633HMC-050 / 100	50 µg or 100 µg
5-mC monoclonal antibody 33D3 (mouse)	MAb-081-100 / 500	100 µg or 500 µg
5-hmC, 5-mC & 5-C DNA standard pack	AF-101-0002	2 µg
Rat IgG	AF-105-0025	25 µg / 25 µl
hMeDIP kit x16	AF-104-0016	16 rxns
MagMeDIP kit x48	mc-magme-048	48 rxns

Conclusions

1. We present a monoclonal antibody against 5-hydroxymethylcytosine that has been demonstrated to be highly specific (no cross-reactivity with C- or mC- containing fragments) with our « dual MeDIP » and hMeDIP assays as well as dotblots and to outperform other antibodies on the market.
2. We have developed an efficient test system that can be used to assess the performance of the antibody, and to compare hydroxymethylated and standard methylated DNA distributions.
3. We expect that our assays and antibodies may represent some of the first systems for evaluating the role of differential methylation in the genome. These tools should be relevant and useful in any cell type.