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#51660

5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb

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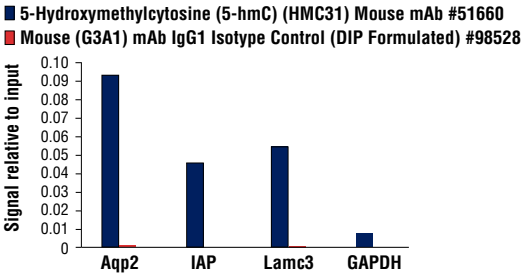
Applications	Species Cross-Reactivity	Isotype
Dot blot, MeDIP, IF-IC	All	Mouse IgG1**
Endogenous		

Background: Methylation of DNA at cytosine residues is a heritable, epigenetic modification that is critical for proper regulation of gene expression, genomic imprinting and mammalian development (1,2). 5-methylcytosine is a repressive epigenetic mark established de novo by two enzymes, DNMT3a and DNMT3b, and it is maintained by DNMT1 (3, 4). 5-methylcytosine was originally thought to be passively depleted during DNA replication. However, subsequent studies have shown that Ten-Eleven Translocation (TET) proteins TET1, TET2, and TET3 can catalyze the oxidation of methylated cytosine to 5-hydroxymethylcytosine (5-hmC) (5). Additionally, TET proteins can further oxidize 5-hmC to form 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), both of which are excised by thymine-DNA glycosylase (TDG), effectively linking cytosine oxidation to the base excision repair pathway and supporting active cytosine demethylation (6,7).

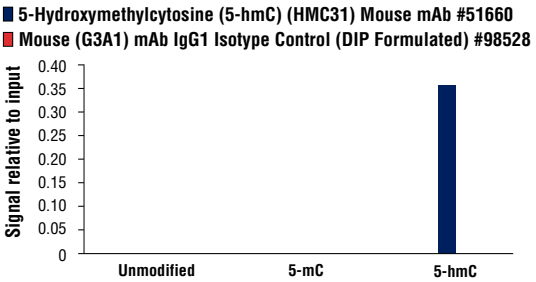
TET protein-mediated cytosine hydroxymethylation was initially demonstrated in mouse brain and embryonic stem cells (5, 8). Since then this modification has been discovered in many tissues, with the highest levels found in the brain (9). While 5-fC and 5-caC appear to be short-lived intermediate species, there is mounting evidence showing that 5-hmC is a distinct epigenetic mark with various unique functions (10,11). The modified base itself is stable in vivo and interacts with various readers including MeCP2 (11,12). The global level of 5-hmC increases during brain development and 5-hmC is enriched at promoter regions and poised enhancers. Furthermore, there is an inverse correlation between levels of 5-hmC and histone H3K9 and H3K27 trimethylation, suggesting a role for 5-hmC in gene activation (12). Lower amounts of 5-hmC have been reported in various cancers including myeloid leukemia and melanoma (13,14).

Specificity/Sensitivity: 5 –Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb recognizes endogenous levels of 5-hmC; however, many cells and tissues contain very low levels of 5-hmC that may fall below the detection limits of this antibody. This antibody has been validated using ELISA, dot blot, and MeDIP assays and shows high specificity for 5-hmC.

Source/Purification: Monoclonal antibody is produced by immunizing animals with 5-hydroxymethylcytidine.



DNA immunoprecipitations were performed with 1 µg of genomic DNA from mouse embryonic stem cells and either 10 µl of 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb or 10 µl of Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) #98528 using SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit #95176. The enriched DNA was quantified by real-time PCR using mouse Aqp2 exon 1 primers, SimpleDIP™ Mouse Intracisternal-A Particle (IAP) LTR Primers #74803, mouse Lamc3 exon 1 primers, and SimpleChIP® Mouse GAPDH Intron 2 Primers #8986. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input DNA, which is equivalent to one.



5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb specificity was determined by DNA immunoprecipitation. DNA IPs were performed with genomic DNA prepared from mouse embryonic stem cells, spiked with DNA containing either unmethylated cytosine, 5-methylcytosine (5-mC), or 5-hydroxymethylcytosine (5-hmC). IPs were performed using SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit #95176. The enriched DNA was quantified by real-time PCR using primers specific to the spiked-in control DNA sequence. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input DNA, which is equivalent to one.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

****Anti-mouse secondary antibodies must be used to detect this antibody.**

Recommended Antibody Dilutions:

Dot blotting	1:1000
MeDIP	1:50
Immunofluorescence (IF-IC)	1:200
IF Protocol:	Special protocol required

For product specific protocols and a complete listing of recommended companion products please see the product web page at www.cellsignal.com

Background References:

(1) Hermann, A. et al. (2004) *Cell Mol Life Sci* 61, 2571-87.
(2) Turek-Plewa, J. and Jagodzinski, P.P. (2005) *Cell Mol Biol Lett* 10, 631-47.
(3) Okano, M. et al. (1999) *Cell* 99, 247-57.
(4) Li, E. et al. (1992) *Cell* 69, 915-26.
(5) Tahiliani, M. et al. (2009) *Science* 324, 930-5.
(6) He, Y.F. et al. (2011) *Science* 333, 1303-7.
(7) Ito, S. et al. (2011) *Science* 333, 1300-3.
(8) Kriaucionis, S. and Heintz, N. (2009) *Science* 324, 929-30.
(9) Globisch, D. et al. (2010) *PLoS One* 5, e15367.
(10) Gao, Y. et al. (2013) *Cell Stem Cell* 12, 453-69.
(11) Mellén, M. et al. (2012) *Cell* 151, 1417-30.
(12) Wen, L. et al. (2014) *Genome Biol* 15, R49.
(13) Delhommeau, F. et al. (2009) *N Engl J Med* 360, 2289-301.
(14) Lian, C.G. et al. (2012) *Cell* 150, 1135-46.

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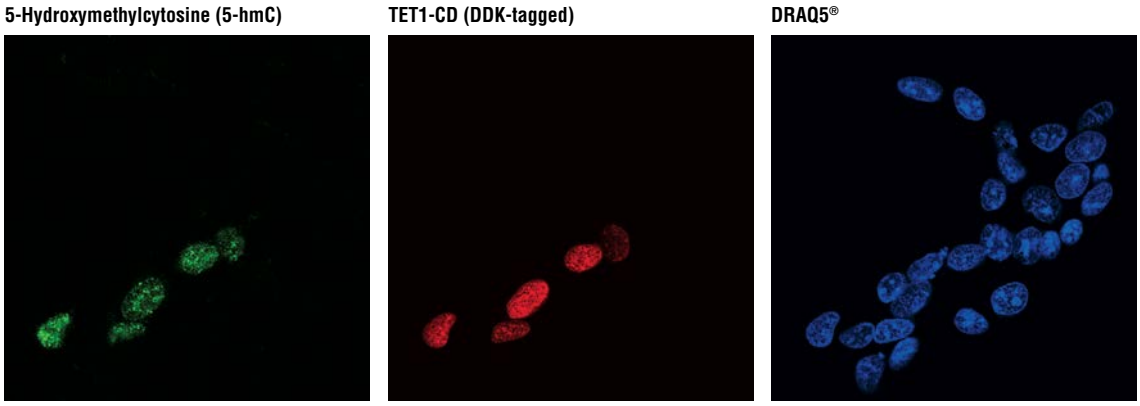
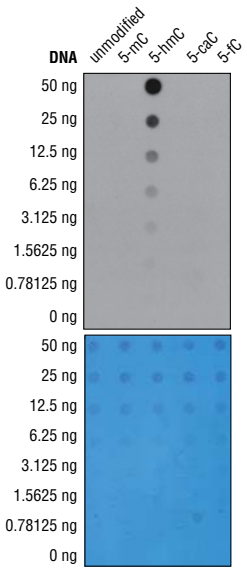
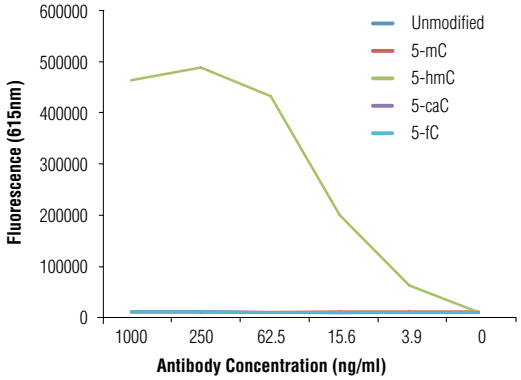
Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.



Confocal immunofluorescent analysis of 293T cells transfected with a construct expressing DDK-tagged TET1 catalytic domain (TET1-CD) using 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb (green) and DYKDDDDK Tag Antibody #2368 (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye). As expected, 293T cells expressing TET1-CD (red) exhibit increased levels of 5-hydroxymethylcytosine (green).



5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb specificity was determined by dot blot. The same sequence of a 387-base pair DNA fragment was generated by PCR using exclusively unmodified cytosine, 5-methylcytosine (5-mC), 5-hydroxymethylcytosine (5-hmC), 5-carboxylcytosine (5-caC), or 5-formylcytosine (5-fC). The respective DNA fragments were blotted onto a nylon membrane, UV cross-linked, and probed with 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb. The upper panel shows the antibody only binding to the DNA fragment containing 5-hmC, while the lower panel shows the membrane stained with methylene blue.



5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb specificity was determined by ELISA. The antibody was titrated against a single-stranded DNA oligo containing either unmodified cytosine or differentially modified cytosine (5-mC, 5-hmC, 5-caC, 5-fC). As shown in the graph, the antibody only binds to the oligo containing 5-hmC.

DNA Dot Blot Protocol

A. Buffers and Reagents

1. **20X Saline Sodium Citrate (SSC) Buffer:** 3.0 M NaCl, 0.3 M Sodium Citrate, pH to 7.0.
2. **10X SSC Buffer:** Dilute 20X SSC buffer 1:2.
3. **2X DNA Denaturing Buffer:** 200 mM NaOH, 20 mM EDTA.
4. **Nuclease-Free Water:** (#12931)
5. **Blotting Membrane:** This protocol has been optimized for positively charged nylon membranes.
6. **96-Well Dot Blot Apparatus**
7. **10X Tris Buffered Saline with Tween® 20 (TBST):** (#9997) To prepare 1 L 1X TBST: add 100 ml 10X TBST to 900 ml dH_20, mix.
8. **Nonfat Dry Milk:** (#9999)
9. **Blocking Buffer:** 1x TBST with 5% w/v nonfat dry milk; for 150 ml, add 7.5 g nonfat dry milk to 150 ml 1X TBST and mix well.
10. **Bovine Serum Albumin (BSA):** (#9998)
11. **Primary Antibody Dilution Buffer:** 1X TBST with 5% BSA; for 20 ml, add 1.0 g BSA to 20 ml 1X TBST and mix well.
14. **Secondary Antibody Conjugated to HRP:** anti-rabbit (#7074); anti-mouse (#7076).
13. **Detection Reagent:** LumiGLO® chemiluminescent reagent and peroxide (#7003) or SignalFire™ ECL Reagent (#6883)

B. Dot Blot

Note: This protocol is written for spotting fragmented, purified genomic DNA (titration of 1000 ng, 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, and 15.625 ng) onto a positively charged nylon membrane using a 96-well dot blotting apparatus. Depending on the source and type of DNA, more or less DNA may be required for detection with the antibody.

Before Starting:

- Purify genomic DNA using a genomic DNA purification protocol or kit and sonicate genomic DNA to generate fragments between 200 and 500 bp. DNA fragment size can be analyzed by gel electrophoresis on a 1% agarose gel with a 100 bp DNA marker.
- Cut a piece of nylon membrane to the size of the dot blot manifold.
- Wet nylon membrane with 10x SSC Buffer.
- Dry membrane by placing it in 96-well dot blot apparatus and applying vacuum.

1. Dilute fragmented genomic DNA to 100 ng/µl in 100 µl of nuclease-free water. Then denature DNA by adding 100 µl of 2X DNA Denaturing Buffer and incubating at 95°C for 10 min.
2. Add 200 µl of 20X SSC buffer and immediately chill on ice for 5 min.
3. Add 100 µl of nuclease-free water to bring DNA solution to a final volume of 500 µl with a DNA concentration of 20 ng/µl.
4. Set up a series of six 2-fold dilutions by adding 250 µl of the DNA solution, starting with the DNA solution in Step 3, to 250 µl of nuclease-free water. This will generate seven DNA samples containing 250 µl DNA at concentrations of 20 ng/µl, 10 ng/µl, 5 ng/µl, 2.5 ng/µl, 1.25 ng/µl, 0.625 ng/µl, and 0.3125 ng/µl.
5. Apply 50 µl of each of the seven dilution samples into separate wells of the 96-well dot blot apparatus, leaving the last well for nuclease-free water only. The amount of DNA added to each well should then be 1000 ng, 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 ng, 15.625 ng and 0 ng respectively. Apply gentle vacuum pressure to draw solution through the membrane. Nylon membrane should be mostly dry before step 6.
6. Remove nylon membrane from the 96-well dot blot apparatus and wrap in plastic wrap.
7. UV cross-link nylon membrane at 1200 J/m^2.

C. Membrane Blocking and Antibody Incubation

1. Incubate membrane in 25 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
2. Wash membrane three times for 5 min each with 15 ml of 1X TBST.
3. Incubate membrane and primary antibody (at the appropriate dilution and diluent as recommended in the antibody product datasheet) in 10 ml primary antibody dilution buffer, with gentle agitation overnight at 4°C.
4. Wash three times for 5 min each with 15 ml of 1X TBST.
5. Incubate membrane with the species appropriate HRP-conjugated secondary antibody (#7074 Anti-rabbit IgG, HRP-linked Antibody or #7076 Anti-mouse IgG, HRP-linked Antibody) at 1:2000 in 10 ml of blocking buffer with gentle agitation for 1 hr at room temperature.
6. Wash membrane three times for 5 min each with 15 ml of 1X TBST.
7. Proceed with detection (Section D)

D. Detection of DNA

1. Incubate membrane with 10 mL of LumiGLO® (0.5 ml 20x LumiGLO® #7003, 0.5 ml 20x Peroxide, and 9.0 ml purified water) or 10 ml SignalFire™ #6883 (5 ml Reagent A, 5 ml Reagent B) with gentle agitation for 1 min at room temperature.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10 sec exposure should indicate the proper exposure time.

NOTE: Due to the kinetics of the detection reaction, signal is most intense immediately following incubation and declines over the following 2 hr.

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Immunofluorescence (Immunocytochemistry)

A. Solutions and Reagents

NOTE: Prepare solutions with reverse osmosis deionized (RODI) or equivalently purified water.

Stock Solutions:

1. **20X Phosphate Buffered Saline (PBS):** (#9808) To prepare 1L 1X PBS: add 50 ml 20X PBS to 950 ml dH2O, mix. Adjust pH to 8.0.
2. **Ethanol,** 70% solution, deionized.
3. 1.5 M **Hydrochloric acid**.
4. **Blocking Buffer** (1X PBS / 5% normal serum / 0.3% Triton™ X-100): To prepare 10 ml, add 0.5 ml normal serum from the same species as the secondary antibody (e.g., Normal Goat Serum (#5425)) and 0.5 mL 20X PBS to 9.0 mL dH2O, mix well. While stirring, add 30 µl Triton™ X-100.
5. **Antibody Dilution Buffer** 1X PBS / 1% BSA / 0.3% Triton™ X-100): To prepare 10 ml, add 30 µl Triton™ X-100 and 0.5 mL 20X PBS to 9.5 mL dH2O. Mix well then add 0.1 g BSA (#9998), mix.
6. **Recommended Fluorochrome-conjugated Anti-Mouse secondary antibodies:**

   Anti-Mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate) #4408

   Anti-Mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 555 Conjugate) #4409

   Anti-Mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 594 Conjugate) #8890

   Anti-Mouse IgG (H+L), F(ab')2 Fragment (Alexa Fluor® 647 Conjugate) #4410
7. **Prolong® Gold AntiFade Reagent** (#9071), **Prolong® Gold AntiFade Reagent with DAPI** (#8961).

B. Specimen Preparation - Cultured Cell Lines (IF-IC)

NOTE: Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or on coverslips.

1. Aspirate media, cover cells completely with cold 70% ethanol.
2. Allow cells to fix for 5 minutes at room temperature.
3. Aspirate fixative, rinse three times in 1X PBS for 5 minutes each.
4. Add 1.5M HCl and incubate for 30 minutes at room temperature.
5. Aspirate HCl and rinse two times in 1X PBS for 5 minutes each.
6. Proceed with Immunostaining section C.

C. Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in Blocking Buffer for 60 minutes.
2. While blocking, prepare primary antibody by diluting as indicated on datasheet in Antibody Dilution Buffer.
3. Aspirate blocking solution, apply diluted primary antibody.
4. Incubate overnight at 4°C.
5. Rinse three times in 1X PBS for 5 minutes each.
6. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for 1–2 hours at room temperature in dark.
7. Rinse three times in 1X PBS for 5 minutes each.
8. Mount samples in an appropriate antifade reagent such as Prolong® Gold Antifade Reagent (#9071) or Prolong® Gold AntiFade Reagent with DAPI (#8961).
9. For best results, allow mountant to cure overnight at room temperature. For long-term storage, store slides flat at 4°C protected from light.

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