Store at -20°C

DNA Cytosine Modification Antibody Sampler Kit

#97763

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www.cellsignal.com/support

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orders@cellsignal.com

New 08/17

For Research Use Only. Not For Use In Diagnostic Procedures.

Products Included	Product #	Quantity	Isotype/Source
5-Methylcytosine (5-mC) (D3S2Z) Rabbit mAb	28692	20 µl	Rabbit IgG
5-Hydroxymethyl-cytosine (5-hmC) (HMC31) Mouse mAb	51660	20 µl	Mouse IgG1
5-Carboxylcytosine (D7S8U) Rabbit mAb	36836	20 µl	Rabbit IgG
5-Formylcytosine (5-fC) (D5D4K) Rabbit mAb	74178	20 µl	Rabbit IgG
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl	Goat
Anti-mouse IgG, HRP-linked Antibody	7076	100 µl	Goat

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.*

Recommended Antibody Dilutions:

Dot blotting	1:1000

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

Description: The DNA Cytosine Modification Antibody Sampler Kit provides an economical means of detecting the levels of cytosine modifications in DNA by dot blot using antibodies against 5-methylcytosine, 5-hydroxymethylcytosine, 5-formylcytosine, and 5-carboxylcytosine.

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 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).

Background References:

(1) Hermann, A. et al. (2004) *Cell Mol Life Sci* 61, 2571-87.

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(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.

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(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.

Specificity/Sensitivity: 5-Methylcytosine (5-mC) (D3S2Z) Rabbit mAb recognizes endogenous levels of 5-methylcytosine. 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb recognizes endogenous levels of 5-hydroxymethylcytosine. 5-Formylcytosine (5-fC) (D5D4K) Rabbit mAb recognizes transfected levels of 5-formylcytosine. 5-Caroxylcytosine (5-caC) (D7S8U) Rabbit mAb recognizes transfected levels of 5-methylcytosine. These antibodies have been validated using ELISA and dot blot, and cross-reactivity was not observed with other marks. Many cells and tissues contain very low endogenous levels of 5-hmC, 5-fC, and 5-caC that may fall below the detection limits of these antibodies.

Source/Purification: Monoclonal antibodies are produced by immunizing animals with 5-methylcytidine, 5-hydroxymethylcytidine, 5-formyl-2'-deoxycytosine, or 5-carboxylcytidine.

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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 1 ng/µl in 100 ul 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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