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#56593

N6-Methyladenosine (m6A) (D9D9W) Rabbit mAb

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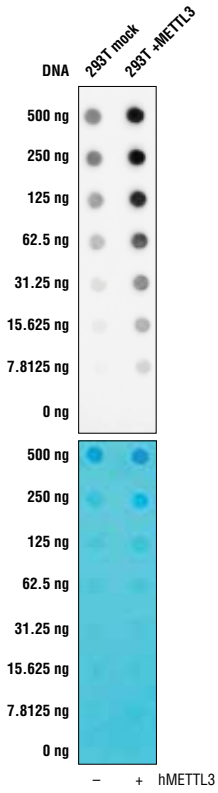
Applications	Species Cross-Reactivity	Isotype
Dot Blot Endogenous	All	Rabbit IgG*

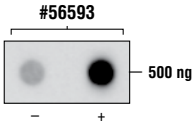
Background: N6-methyladenosine (m6A) is a post-transcriptional modification found in various RNA subtypes. While the presence of m6A in RNA was described decades ago, the lack of tools has made interrogating the epitranscriptomic landscape challenging (1,2). With the emergence of new technologies such as miCLIP and NG-RNA-seq, researchers have been able to show that m6A is a biologically relevant mark in mRNA that is enriched in 3' UTRs and stop codons (3,4). The m6A writer complex consists of a core heterodimer of methyltransferase-like protein 3 (METTL3) and methytransferase-like protein 14 (METTL14), and the additional regulatory proteins Virlizer/VIRMA and Wilms tumor 1-associated protein (WTAP) (5). METTL3 is the catalytic methyltransferase subunit and METTL14 is the target recognition subunit that binds to RNA (6). The Virilzer/VIRMA protein directs m6A methylation to the 3' UTRs and stop codons, and WTAP targets the complex to nuclear speckles, which are sites of RNA processing (7). Less is known about readers and erasers of m6A, and while the fat mass and obesity-associated protein FTO was the first discovered m6A demethylase, subsequent studies demonstrated that this enzyme may prefer the closely related m6Am mark *in vivo* (8,9). ALKBH5 was later shown to be a bona fide m6A demethylase enzyme, contributing to the idea that the m6A modification is dynamically regulated (10). Readers of the m6A mark include the YTH protein family, which can bind to m6A and influence mRNA stability and translation efficiency (3,11-13). The m6A mark and machinery have been shown to regulate a variety of cellular functions, including RNA splicing, translational control, pluripotency and cell fate determination, neuronal function, and disease (1, 14-17). The m6A writer complex has been linked to various cancer types including AML and endometrial cancers (18,19). Additionally, m6A has been implicated in resistance to chemotherapy (20).

Specificity/Sensitivity: N6-Methyladenosine (m6A) (D9D9W) Rabbit mAb recognizes endogenous levels of N6-methyladenosine (m6A). This antibody has been validated using ELISA and dot blot assays and shows high specificity for m6A. This antibody does not cross-react with unmodified adenosine, N6-dimethyladenosine, N1-methyladenosine, or 2'-O-methyladenosine..

Source/Purification: Monoclonal antibody is produced by immunizing animals with N6-methyladenosine.



Total RNA purified from 293T cell extracts, either mock transfected (-) or transfected with a DNA construct expressing full-length human METTL3 (hMETTL3; +), were blotted onto a nylon membrane, UV cross-linked, and probed with N6-Methyladenosine (m6A) (D9D9W) Rabbit mAb. The top panel shows the antibody detecting more methylated adenosine in cells overexpressing METTL3, while the bottom panel shows the membrane stained with methylene blue.



Poly(A)+ RNA purified from 293T cell extracts, either mock transfected (-) or transfected with a DNA construct expressing full-length human METTL3 (+), were blotted onto a nylon membrane, UV cross-linked, and probed with N6-Methyladenosine (m6A) (D9D9W) Rabbit mAb.

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

Recommended Antibody Dilutions:

Dot blotting	1:1000

This antibody has been shown by an independent laboratory to work in RNA-IP-seq. Please use at an assay-dependent dilution.

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) Meyer, K.D. and Jaffrey, S.R. (2017) *Annu Rev Cell Dev Biol* 33, 319-42.

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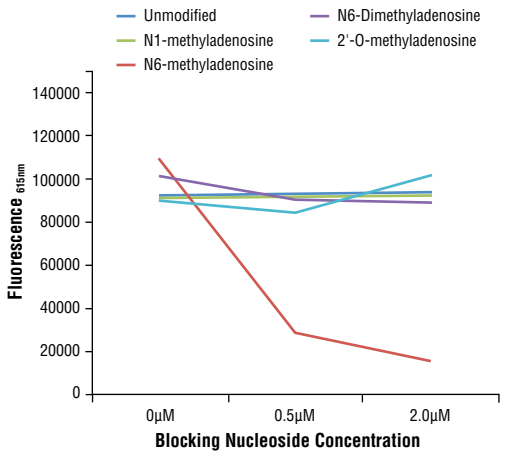
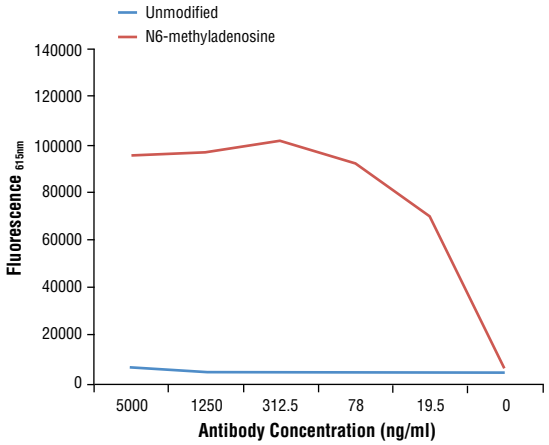
Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **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.



Specificity of N6-Methyladenosine (m6A) (D9D9W) Rabbit mAb was determined by competitive ELISA. The graph depicts the binding of the antibody to a pre-coated m6A olgionucleotide in the presence of increasing concentrations of differentially modified adenosine. As shown in the graph, antibody binding is only blocked by free m6A nucleoside.



Specificity of N6-Methyladenosine (m6A) (D9D9W) Rabbit mAb was determined by ELISA. The antibody was titrated against an RNA oligo containing either unmodified adenosine or N6-methylated adenosine (m6A). As shown in the graph, the antibody only binds to N6-Methyladenosine (m6A).

RNA 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. **4X RNA Denaturing Buffer:** 16.4 M Formamide, 2.8 M Formaldehyde, 26.6 mM MOPS Buffer (6.7 mM Sodium Acetate, 1.3 mM EDTA, 1.3 mM EGTA
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 dH2O; mix
8. Nonfat Dry Milk: (#9999)
9. **Blocking Buffer/Secondary Antibody Dilution 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
12. **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 either purified total RNA or poly A-purified mRNA (titration of 2 µg, 1 µg, 500 ng, 250 ng, 125 ng, 62.5 ng, and 31.25 ng) onto a positively charged nylon membrane using a 96-well dot blotting apparatus. Depending on the source of the RNA, more or less RNA may be required for detection with the antibody.

Before Starting:

- RNA is sensitive to degradation by RNases, which can affect sample integrity. It is recommended that all surfaces and equipment undergo RNase decontamination.
- Purify total RNA and/or mRNA from cell pellet using an RNA isolation kit. Assess total RNA quality by gel electrophoresis on a 1% agarose gel. The 28S and 18S RNA should present as distinct bands (see Figure 1). Smearing indicates RNA degradation.
- Cut a piece of nylon membrane to fit the size of the dot blot manifold.
- Wet nylon membrane with 10X SSC Buffer.
- Dry membrane by placing it in a 96-well dot blot apparatus and applying vacuum.

1. Dilute RNA to 160 ng/µL in 50 µl of nuclease free water. Denature RNA by adding 16.5 µL of 4X RNA Denaturing Buffer and incubate at 65° C for 5 min.
2. Add 66.5 µL of 20X SSC buffer and immediately chill on ice for 5 min.
3. Add 67 µL of nuclease-free water to bring RNA solution to a final volume of 200 µL with an RNA concentration of 40 ng/µl.
4. Set up a series of six, 2-fold dilutions by adding equal volume of the RNA solution, starting with the RNA solution in Step 3, to nuclease-free water. This will generate seven RNA samples with concentrations of 40, 20, 10, 5, 2.5, 1.25, and 0.625 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 RNA added to each well should then be 2 µg, 1 µg, 500 ng, 250 ng, 125 ng, 62.5 ng, 31.25 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 the nylon membrane from the 96-well dot blot apparatus and wrap in plastic wrap.
7. UV cross-link nylon membrane at 1200 J/m2.
8. Repeat Step 7 for a second round of UV cross-linking.

C. Membrane Blocking and Antibody Incubation

Optional: To normalize sample loading using methylene blue, apply stain before Section C, Step 1 and capture an image. Rinse blots three times for 5 min each with 15 mL dH2O. Stain does not affect antibody binding or detection.

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 RNA

1. Incubate membrane with 10 mL of LumiGLO® (0.5 mL of 20X LumiGLO®, 0.5 ml 20X Peroxide #7003 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 capture images using a chemiluminescent-sensitive detection method (film exposure or digital imager).

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