Product Manual

N^6-Methyladenosine (m6A) ELISA Kit

Catalog Number

MET-5097 **96 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
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Introduction

N^6-Methyladenosine (m6A) is a nucleoside modification in RNA (and in DNA at extremely low levels) found in most eukaryotes as well as some viruses. m6A is also found in tRNA, rRNA, small nuclear RNA (snRNA) as well as several long non-coding RNA (ncRNA). A large m6A methyltransferase complex directs methylation of adenosine. The complex includes METTL3 which binds s-adenosylmethionine (SAM). Additional components of methyltransferase complex in mammals include METTL14, Wilms tumor 1 associated protein (WTAP) and KIAA1429. More recently, fat mass and obesity-associated protein (FTO) as well as demethylase alkB homolog 5 (ALKBH5) have been identified as m6A demethylases.

Links between m6A and numerous cancer types have been identified such as stomach prostate, breast, pancreatic, and kidney cancer, as well as mesothelioma, sarcoma, and leukemia. Reduction in METTL3 levels has been shown to cause apoptosis of cancer cells and reduce invasiveness of cancer cells, while increased ALKBH5 activity by hypoxia was shown to cause increase cancer stem cell numbers. m6A has also been implicated energy homeostasis and obesity regulation since FTO is a key regulator of energy metabolism and obesity. Furthermore, neurodegenerative diseases may be influenced by m6A since dopamine signaling was shown to be FTO dependent. ZC3H13, yet another member of the m6A methyltransferase complex, greatly inhibited growth of colorectal cancer cells when knocked down.

Additionally, m6A affects viral infectivity. RNA viruses such as SV40, adenovirus, herpes virus, Rous sarcoma virus, and influenza virus contain m6A methylation on virus genomic RNA. Regulators of m6A control the efficiency of infection and replication of RNA viruses like human immunodeficiency virus (HIV), hepatitis C virus (HCV), and Zika virus (ZIKV). m6A and its cognate factors play crucial roles in regulating virus life cycle and host-viral interactions.

The m6A ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of m6A in urine, serum, cell or tissue samples. The quantity of m6A in unknown samples is determined by comparing its absorbance with that of a known m6A standard curve. The kit has detection sensitivity limit of 80 ng/mL m6A. Each m6A ELISA Kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

Assay Principle

The m6A ELISA kit is a competitive ELISA for the quantitative measurement of m6A. The unknown m6A samples or m6A standards are first added to an m6A conjugate preabsorbed microplate. After a brief incubation, an anti-m6A monoclonal antibody is added, followed by an HRP conjugated secondary antibody. The m6A content in unknown samples is determined by comparison with a predetermined m6A standard curve.

Related Products

1. MET-5090: Adenosine Assay Kit
2. MET-5092: Inosine Assay Kit
3. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)

4. STA-324: OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP Sites)
5. STA-380: Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation)
6. STA-672: S-Adenosylmethionine (SAM) ELISA Kit
7. STA-820-C: OxiSelect™ Aldehyde-Induced DNA Damage ELISA Combo Kit (Ethenoadenosine / Ethenocytidine Quantitation)
8. STA-825: OxiSelect™ Nitrosative DNA/RNA Damage ELISA Kit (8-Nitroguanine Quantitation)

Kit Components

Box 1 (shipped at room temperature)

1. 96-well Protein Binding Plate (Part No. 231001): One strip well 96-well plate.
2. Anti-m6A Antibody (500X) (Part No. 50971C): One 10 µL vial.
3. Secondary Antibody, HRP Conjugate (Part No. 230003): One 20 µL vial.
4. Assay Diluent (Part No. 310804): One 50 mL bottle.
5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
7. Stop Solution (Part. No. 310808): One 12 mL bottle.
8. m6A Standard (Part No. 50972C): One 25 µL vial of 0.5 mg/mL N6-methyladenosine.

Box 2 (shipped on blue ice packs)

1. m6A Conjugate (100X) (Part No. 50973D): One 100 µL vial.
2. 100X Conjugate Diluent (Part No. 281603): One 300 µL vial.

Materials Not Supplied

1. m6A containing samples such as urine, serum, plasma, or RNA samples extracted from cells or tissues
2. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
3. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
4. Multichannel micropipette reservoir
5. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)
6. RNA extraction kit
7. Nuclease P1
8. Alkaline Phosphatase

Storage

Upon receipt, aliquot and store m6A standard at -20°C and the m6A Conjugate (100X) at -80ºC avoiding multiple freeze/thaw cycles. Store all other components at 4ºC.

Preparation of Reagents

- m6A Conjugate Coated Plate:

  Note: The m6A Conjugate coated wells are not stable and should be used within 24 hrs after coating. Only coat the number of wells to be used immediately.

  1. Immediately before use, prepare 1X Conjugate Diluent by diluting the 100X Conjugate Diluent in 1X PBS. Example: Add 50 µL to 4.95 mL of 1X PBS.
  2. Immediately before use, prepare 1X m6A Conjugate by diluting the 100X m6A Conjugate in 1X Conjugate Diluent. Example: Add 50 µL of 100X m6A Conjugate to 4.950 mL of 1X Conjugate Diluent.
  3. Add 100 µL of the 1X m6A Conjugate to each well to be tested and incubate at 37ºC for two hours or overnight at 4ºC. Remove the m6A Conjugate coating solution and wash twice with 1X PBS. Blot plate on paper towels to remove excess fluid. Add 200 µL of Assay Diluent to each well and block for 1 hr at room temperature on an orbital shaker. Transfer the plate to 4ºC and remove the Assay Diluent **immediately before use.**

- 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity.
- Anti-m6A Antibody and Secondary Antibody: Immediately before use dilute the Anti-m6A Antibody 1:500 and Secondary Antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Preparation of Standard Curve

1. Use the provided stock m6A Standard 0.5 mg/mL solution to prepare a series of the remaining m6A standards according to Table 1 below.

Standard Tubes	0.5 mg/mL m6A Standard (µL)	Assay Diluent (µL)	m6A (ng/mL)	m6A (µM)
1	5	495	5000	17.8
2	250 of Tube #1	250	2500	8.9
3	250 of Tube #2	250	1250	4.5
4	250 of Tube #3	250	625	2.2
5	250 of Tube #4	250	313	1.1
6	250 of Tube #5	250	156	0.56
7	250 of Tube #6	250	78	0.28
8	0	250	0	0

Table 1. Preparation of m6A Standards.

Preparation of Samples

- Urine: Samples containing precipitates should be centrifuged at 3000 g for 10 minutes, or filtered through 0.45 µm filter, prior to use in the assay. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.
- Serum: Avoid hemolyzed and lipemic blood samples. Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Aliquot samples for testing and store at -80ºC. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.

  Note: This assay is <u>not</u> compatible with mouse serum due to high levels of mouse IgG that will cross react with the secondary antibody.
- Plasma: Avoid hemolyzed and lipemic blood samples. Collect blood with heparin or citrate and centrifuge at 2000 x g and 4ºC for 10 minutes. Remove the plasma layer and store on ice. Avoid disturbing the white buffy layer. Aliquot samples for testing and store at -80ºC. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.

  Note: This assay is <u>not</u> compatible with mouse plasma due to high levels of mouse IgG that will cross react with the secondary antibody.
- Cells or Tissues: RNA must first be isolated before assaying.
  1. Purify RNA from cell or tissue samples by a desired method or commercial RNA Extraction kit.
  2. Dissolve purified RNA in nuclease free water at 1-5 mg/mL.
  3. Remove any RNA secondary structure by incubating the sample at 95ºC for 5 minutes and rapidly chilling on ice.
  4. Digest RNA sample to nucleosides by incubating the denatured RNA with 5-20 units of nuclease P1 (previously reconstituted in the manufacturer's recommended buffer) for 2 hrs at 37ºC in a final concentration of 20 mM Sodium Acetate, pH 5.2.
  5. Add 5-10 units of alkaline phosphatase (previously reconstituted in the manufacturer's recommended buffer) plus sufficient Tris buffer to a final concentration of 100 mM Tris, pH 7.5, and incubate for 1 hr at 37ºC.
  6. Centrifuge the reaction mixture for 5 minutes at 6000 x g and collect the supernatant for use in the ELISA.

  Note: Based on massively parallel sequencing analysis (Dominissini et al, 2012; see ref 2), there are about 0.5 ng m6A per µg of normally methylated human mRNA. Therefore, we recommend using at least 20 µg of digested normally methylated RNA per assay.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each m6A sample including unknown and standard should be assayed in duplicate.

2. Add 50 µL of unknown sample or m6A standards to the wells of the m6A Conjugate coated plate. Incubate at room temperature for 5 minutes on an orbital shaker.
3. Add 50 µL of the diluted anti-m6A antibody to each well, incubate at room temperature for 1 hour on an orbital shaker.
4. Wash microwell strips 3 times with 250 µL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
5. Add 100 µL of the diluted Secondary Antibody-HRP Enzyme Conjugate to all wells.
6. Incubate at room temperature for 1 hour on an orbital shaker.
7. Wash microwell strips 3 times according to step 4 above. Proceed immediately to the next step.
8. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.

   Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.
9. Stop the enzyme reaction by adding 100 µL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
10. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figures demonstrate typical m6A ELISA results. One should use the data below for reference only. This data should not be used to interpret actual results.

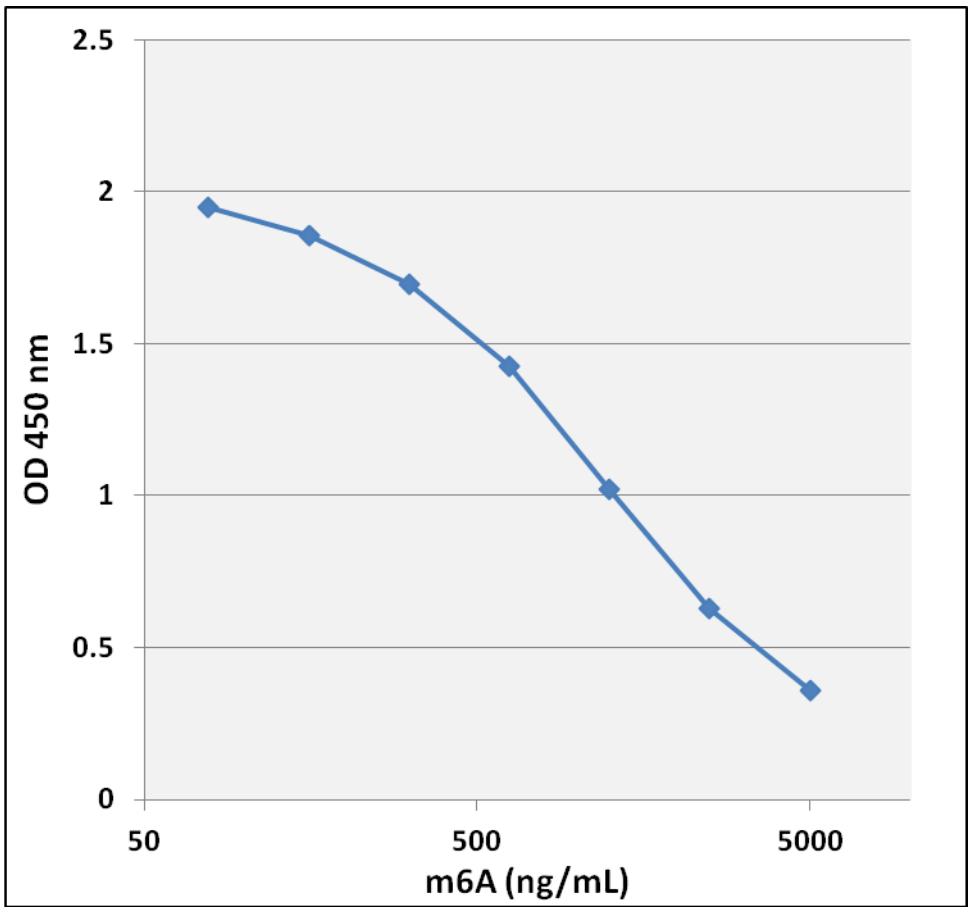

Figure 1: m6A ELISA Standard Curve.

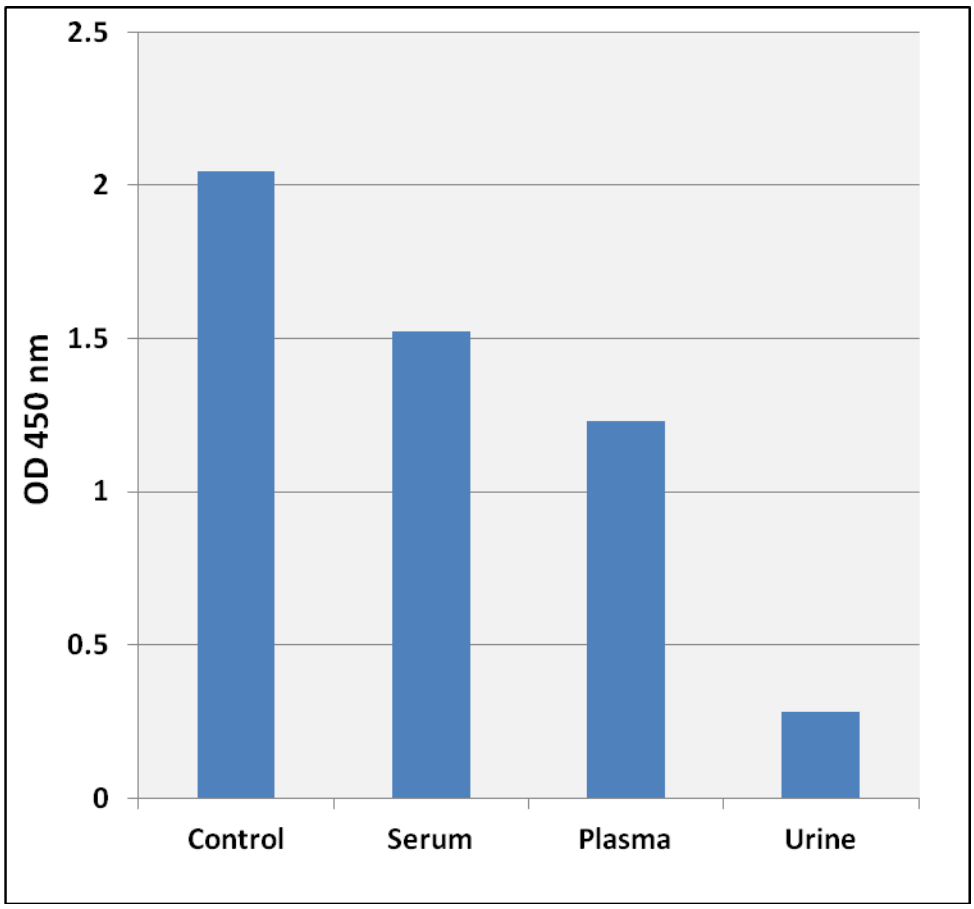

Figure 2: m6A Levels in human Serum, Plasma, or Urine compared to Negative Control (Assay Diluent). Undiluted human samples were tested according to the Assay Protocol.

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