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ab233493
Urine m6A Assay Kit

For detecting total urinary m^6A levels, resulting from whole body turnover or degradation of DNA/RNA containing m^6A, using urine from humans and animals.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Urine m6A Assay Kit (ab233493) is a complete set of optimized buffers and reagents to colorimetrically quantify m^6A in urine using an inhibitory competitive immunoassay method. It is suitable for detecting total urinary m^6A levels, resulting from whole body turnover or degradation of DNA/RNA containing m^6A, using urine from humans and animals.

In this ELISA-like inhibitory competitive immunoassay, urine samples and the m^6A standard are first incubated with a m^6A antibody solution and then transferred to strip wells coated with m^6A polynucleotide. The well is washed to remove any unbound reagents after incubation and then a detection antibody is added to generate a signal that can be measured colorimetrically by reading the absorbance in a microplate spectrophotometer. Because m^6A in the urine sample inhibits the binding of m^6A antibody to m^6A coated on the well, higher concentrations of m^6A in the urine sample lead to a reduced binding of the antibody to the m^6A on the well. Therefore, the signal or OD intensity measured from the well will be inversely proportional to the amount of m^6A in the urine sample and the amount of m6A in the urine sample can be quantified by a comparison with a predetermined m^6A standard.

This kit contains a unique binding solution allowing nucleic acids >70 nts to be tightly bound to the wells, which enables quantification of m6A from both mRNA and nc-RNA such as tRNA, rRNA and snRNA.

Prepare antibody solution.

Add urine sample or M^{6}A standard plus antibody solution to prep plate. Meanwhile, bind m^{6}A to assay plate for 90 minutes then wash wells.

Incubate prep plate for 60 minutes at 37°C.

Transfer solutions from prep plate to assay plate.

Wash assay wells, then add detection antibody and enhancer solution.

Add color developing solution followed by stop solution, then measure absorbance.

2. Materials Supplied and Storage

Store m^{6}A Standard, Negative Control, m^{6}A Assay Solution, Detection Antibody and Enhancer Solution at –20°C away from light. Store 10X Wash Buffer, Capture Antibody, Developer Solution, and Plate 1 at 4°C away from light. Store Binding Solution, Stop Solution and Plate 2 at room temperature away from light. Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

Item	48 tests Quantity	96 tests Quantity	Storage temperature (before prep)
10X Wash Buffer	14 mL	28 mL	4°C
Binding Solution	5 mL	10 mL	RT
m^{6}A Standard, 2 µg/mL	50 µL	100 µL	-20°C
Negative Control, 50 µg/mL	10 µL	20 µL	-20°C
m^{6}A Assay Solution, 500X	10 µL	20 µL	-20°C
Capture Antibody, 1000X	4 µL	8 µL	4°C
Detection Antibody, 2000X	4 µL	8 µL	-20°C
Enhancer Solution	4 µL	8 µL	-20°C
Developer Solution	5 mL	10 mL	4°C
Stop Solution	5 mL	10 mL	RT
Plate 1 (Assay Plate, flat bottom)	1 (6 strips)	1 (12 strips)	4°C
Plate 2 (Sample Preparation Plate, round bottom)	1 (6 strips)	1 (12 strips)	RT

Δ Note The Negative Control is an RNA containing no m^{6}A. The m^{6}A Assay Solution is m^{6}A oligos and is normalized to have 100% of m^{6}A.

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microplate reader capable of reading absorbance at 450 nm.
- Incubator for 37°C incubation.
- Plate seal or Parafilm M.
- Distilled water.
- 1X TE buffer pH 7.5 to 8.0.
- Urine sample.

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

Keep each diluted solution, except diluted 1X Wash Buffer, on ice until use. Any remaining diluted solutions other than diluted should be discarded if not used within the same day.

5.1 10X Wash Buffer

5.1.1 For a 48-reaction size kit, prepare diluted 1X Wash Buffer by adding 13 mL of 10X Wash Buffer to 117 mL of distilled water and adjust pH to 7.2-7.5.

5.1.2 For the 96-reaction size kit, add 26 mL of 10X Wash Buffer to 234 mL of distilled water and adjust pH to 7.2-7.5.

Δ Note Check if 10X Wash Buffer contains salt precipitates before use. If so, briefly warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved.

Δ Note Diluted 1X Wash Buffer can now be stored at 4°C for up to six months.

5.2 Binding Solution

Ready to use as supplied.

5.3 m⁶A Standard, 2 µg/mL

Ready to use as supplied.

5.4 Negative Control, 50 µg/mL

Ready to use as supplied.

5.5 m⁶A Assay Solution, 500X

Prepare diluted 1X m⁶A Assay solution by diluting 500X m⁶A Assay Solution at 1:500 ratio (1 µL of m⁶A Assay solution + 500 µL of BS Binding Solution). About 100 µL of diluted m⁶A Assay solution will be required for each assay well.

5.6 Capture Antibody, 1000X

Prepare diluted Capture Antibody Solution by diluting Capture Antibody with diluted WB 1X Wash Buffer at a ratio of 1:1000 (ex: add 1 µL of Capture Antibody to 1000 µL of

diluted 1X Wash Buffer). About 50 µL of diluted Capture Antibody will be required for each assay well.

5.7 Detection Antibody, 2000X

Prepare diluted Detection Antibody Solution by diluting Detection Antibody with diluted 1X Wash Buffer at a ratio of 1:2000 (ex: add 1 µL of Detection Antibody to 2000 µL of diluted 1X Wash Buffer). About 50 µL of diluted Detection Antibody will be required for each assay well.

5.8 Enhancer Solution, 1000X

Prepare diluted Enhancer Solution by Enhancer Solution with diluted 1X Wash Buffer at a ratio of 1:3000 (ex: add 1 µL of Enhancer Solution to 3000 µL of diluted 1X Wash Buffer). About 50 µL of diluted Enhancer Solution will be required for each assay well.

5.9 Developer Solution

Ready to use as supplied.

5.10 Plate 1 (Assay Plate, flat bottom)

Ready to use as supplied.

5.11 Plate 2 (Sample Preparation Plate, round bottom)

Ready to use as supplied.

6. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

1. First, dilute m^{6}A Standard Solution to 1 ng/µL (4 µL of m^{6}A Standard Solution + 4 µL of 1X TE) and 0.1 ng/µL (1 µL of m^{6}A Standard Solution + 19 µL of 1X TE).
2. Then, further prepare five different concentrations with the diluted MS and 1X TE into 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng/µL, according to the following dilution chart:

Standard	m^{6}A Standard Solution (1 ng/µL)	m^{6}A Standard Solution (0.1 ng/µL)	1X TE	Resulting m^{6}A Standard Concentration
1	-	1.0 µL	4.0 µL	0.02 ng/µL
2	-	2.0 µL	2.0 µL	0.05 ng/µL
3	-	3.0 µL	0.0 µL	0.1 ng/µL
4	1.0 µL	-	4.0 µL	0.2 ng/µL
5	1.5 µL	-	1.5 µL	0.5 ng/µL
6	3.0 µL	-	0.0 µL	1.0 ng/µL

7. Sample Preparation

General sample information:

Input Urine Volume:

Urine amount can range from 1 to 20 µL per assay. An optimal amount is 5 µL per assay. Clear urine samples can be directly used for the assay. Centrifugation at 2500-3000 g for 10 minutes should be required for the samples containing precipitates.

Storage:

Urine sample should be stored at -20°C immediately after collection.

8. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

8.1 m^{6}A Binding:

1. Predetermine the number of assay strip wells from Plate 1 to be required for your experiment. Carefully remove un-needed strip wells from the plate frame and place them back in the bag (seal the bag tightly and store at 4°C).
2. For negative control wells: Add 100 µL of Binding Solution to each negative control well.
3. For no sample control wells: Add 100 µL of diluted m^{6}A Assay solution to each no sample control well.
4. For standard wells: Add 100 µL of Binding and 1 µL of diluted m^{6}A Standard at the different concentrations from 0.02 to 1 ng/µL (as shown in Section 6) to each standard well.
5. For sample wells: Add 100 µL of diluted m^{6}A Assay solution to each no sample control well.
6. Cover Plate 1 with plate seal or Parafilm M and incubate at 37°C for 90 minutes.
7. Meanwhile, predetermine the number of wells required in Plate 2 (Sample Preparation Plate). Cover remaining unused wells with plate seal and set up sample preparation in the subsequent steps (Steps 8 to 12).
8. For negative control wells: Add 50 µL of diluted Capture Antibody, 4 µL diluted 1X Wash Buffer and 1 µL Negative Control to each negative control well.
9. For no sample control wells: Add 50 µL of diluted Capture Antibody and 5 µL diluted 1X Wash Buffer to each control well.
10. For standard wells: Add 50 µL of diluted Capture Antibody and 5 µL diluted 1X Wash Buffer to each standard well.
11. For sample wells: Add 50 µL of diluted Capture Antibody and 5 µL urine sample to each sample well.

8.2 m⁶A Capture:

1. Carefully transfer the solution from each well of Plate 2 to the corresponding well of Plate 1. Plate 2 will no longer be used from this point forward.
2. Cover Plate 1 with a plate seal or Parafilm M and incubate at RT for 60 minutes.
3. Remove the solution from each well.
4. Wash each well three times with 150 µL of the diluted 1X Wash Buffer each time.
5. Add 50 µL of the diluted Detection Antibody to each well, then cover and incubate at room temperature for 30 minutes.
6. Remove the diluted Detection Antibody solution from each well.
7. Wash each well four times with 150 µL of the diluted 1X Wash Buffer each time.
8. Add 50 µL of the diluted Enhancer Solution to each well, then cover and incubate at room temperature for 30 minutes.
9. Remove the diluted Enhancer Solution from each well.
10. Wash each well five times with 150 µL of the diluted 1X Wash Buffer each time.

8.3 Signal Detection:

1. Add 100 µL of Developer Solution to each well and incubate at room temperature for 1 to 10 minutes away from light. Begin monitoring color change in the sample wells and control wells. The Developer Solution will turn blue in the antibody-bound wells.
2. Add 100 µL of Stop Solution to each well to stop the enzyme reaction when color in the standard wells starts turning medium blue. Mix the solution by gently shaking the frame and wait 1-2 min to allow the color reaction to be completely stopped. The color will change to yellow after adding Stop Solution and the absorbance should be read on a microplate reader at 450 nm within 2 to 15 minutes.
   Δ Note If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

9. Data Analysis

To quantify the amount of m^{6}A, first generate a standard curve and plot the OD values against the amount of m^{6}A Standard at each concentration point. Next, determine the slope (OD/ng) of the standard curve using linear regression (Microsoft Excel's linear regression functions are suitable for such calculation) and the most linear part (include at least 4 concentration points) of the standard curve for optimal slope calculation. Now calculate the amount and concentration of m6A in urine sample using the following formula:

$$\text{m6A (ng/mL)\%} = \frac{(\text{No Sample Control OD - NC OD}) - (\text{Sample OD - NC OD})}{\text{Slope x Urine Volume}} * 1000$$

Example Calculation:
Average OD450 of Negative Control (NC) is 0.115
Average OD450 of No Sample control is 0.815
Average OD450 of sample is 0.615
Slope is 0.4 OD/ng
Urine volume is 5 µL

$$\text{m6A (ng/mL)\%} = \frac{0.5 - 0.7}{-0.4 \times 5} * 1000 = 100 \text{ ng/mL}$$

10. Typical Data

Data provided **for demonstration purposes only.**

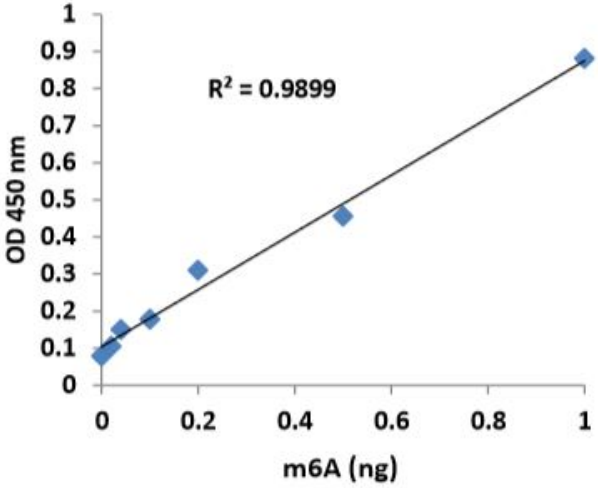

Figure 1. m⁶A standard was added into the assay wells at different concentrations and then measured with Urine m6A Assay Kit (ab233493).

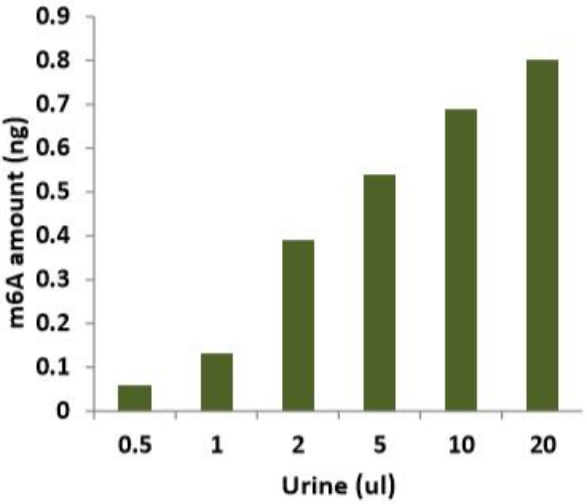

Figure 2. m^{6}A level is quantified from different volumes of human urine using Urine m6A Assay Kit (ab233493).

11. Troubleshooting

Problem	Reason	Solution
No signal or weak signal in the standard wells and the sample wells	Reagents are added incorrectly.	Check if reagents are added in the proper order and if any steps in the protocol may have been omitted by mistake.
	Antibody was not correctly added into the designated wells of Plate 2 (Sample Preparation Plate).	Ensure the antibody at a proper dilution is added into the designated wells of Plate 2 (Sample Preparation Plate).
	The bottom of the well is not completely covered by the Binding Solution.	Ensure the solution coats the bottom of the well by gently tilting from side to side or gently shaking the plate several times.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Insufficient m^6A Assay Solution is added into the assay wells	Ensure that a sufficient amount of m^6A Assay Solution is added into the wells.
	Incorrect absorbance reading.	Check if appropriate absorbance wavelength (450 nm) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and the caps are tightly capped after each opening or use.
No signal or weak signal in only the sample wells	Too much urine sample is used.	Ensure the volume of urine samples added into the wells is within the recommended range. Optimal volume is 5 µL.

No signal or weak signal in only the standard wells	The m6A Standard solution is not properly diluted.	Properly dilute the MS m6A Standard solution to the different concentrations according to the Dilution Chart in Section 6.
High background present in the negative control wells	Insufficient washing of wells.	Check if washing recommendations at each step are performed according to the protocol.
	Contaminated by m6A Assay Solution.	Ensure the well is not contaminated from adding m6A Assay Solution accidentally or from using contaminated tips.
	Over development of color.	Decrease the development time in Step 8.3.1 before adding Stop Solution in Step 8.3.2.
Large variation between replicate wells	Color reaction is not evenly stopped due to an inconsistency in pipetting time or in pipetting volume.	Ensure Developer Solution and Stop Solution is added at the same time between replicates or otherwise maintains a consistent timing in between each addition of solutions.
	Color reaction is not evenly stopped due to an inconsistent order of adding solutions.	Ensure all solutions, particularly Developer Solution and Stop Solution, are added in the same order each time as all other solutions.
	The solutions are not evenly added due to inconsistency in pipetting volume.	Ensure the solution in each pipette tip is equal in the multi-channel pipette. Equilibrate the pipette tip in any solutions before adding them. Ensure the solutions, especially those with small volume (ex: 1 uL) are completely added into the wells.
	Solutions or antibodies were not actually added into the wells.	Do not allow pipette tip to touch the outer edges or inner sides of the wells to prevent solutions from sticking to the surface

Large variation between replicate wells	Did not sufficiently shake the solutions in the wells at Step 8.3.1 and Step 8.3.2.	Gently and evenly shake the plate frame across a flat surface so that the solutions in the wells are better distributed. Do not stir.
	Did not use the same pipette device throughout the experiment.	Use the same multi-channel pipette device throughout the entire experiment, as different pipette devices may have slight variations in performance.
Capture Antibody vial appears to be empty or insufficient in volume	Buffer evaporated due to the very small volumes, resulting in a higher concentrated antibody.	Add 1X PBS buffer into the Capture Antibody vial until you restore the correct, intended volume according to the Kit Contents described in this User Guide. Mix and centrifuge prior to use.

12. Notes

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)