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ab233491
m6A RNA Methylation Assay Kit (Fluorometric)

For detecting m^6A RNA methylation levels using RNA isolated from any species including mammals, plants, fungi, bacteria and viruses in cultured cells, fresh and frozen tissues, paraffin-embedded tissues, plasma/serum samples, and body fluid samples.

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

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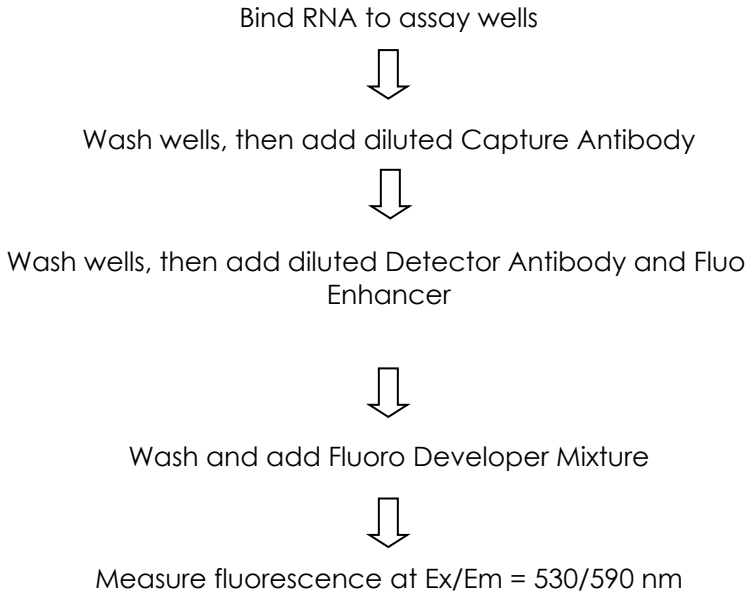
1. Overview

m6A RNA Methylation Assay Kit (Fluorometric) (ab233491) is a complete set of optimized buffers and reagents to fluorometrically quantify methylated N6-methyladenosine (m^6A) in RNA. It is suitable for a direct detection of m^6A RNA methylation status using total RNA isolated from any species such as mammals, plants, fungi, bacteria and viruses.

This kit contains a unique binding solution allowing RNA >70 nts to be tightly bound to the wells, which enables quantification of m^6A from both mRNA and nc-RNA such as tRNA, rRNA and snRNA. The optimized antibody and enhancer solutions allow high specificity to m^6A, with no cross-reactivity to unmethylated adenosine within the indicated concentration range of the sample RNA. Also included are universal positive and negative controls which are suitable for quantifying m^6A from any species.



2. Materials Supplied and Storage

Store Negative Control, Positive Control, Detector Antibody, Enhancer Solution and Fluoro Developer at –20°C away from light. Store Wash Buffer, Capture Antibody, Fluoro Enhancer and 8-Well Assay Strips at 4°C away from light. Store Binding Solution and Dilution Buffer 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
Negative Control, 100 µg/mL	10 µL	20 µL	-20°C
Positive Control, m^{6}A 2 µg/mL	10 µL	20 µL	-20°C
Capture Antibody, 1000 X	5 µL	10 µL	4°C
Detector Antibody, 1000 X	6 µL	12 µL	-20°C
Enhancer Solution	5 µL	10 µL	-20°C
Fluoro Developer	8 µL	16 µL	-20°C
Fluoro Enhancer	8 µL	16 µL	4°C
Dilution Buffer	4 mL	8 mL	RT
8-Well Assay Strips	6	12	4°C

Δ Note The Negative Control is an RNA containing no m^{6}A. The Positive Control is m^{6}A oligos and is normalized to have 100% 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 fluorescence at Ex/Em = 530/590 nm.
- Incubator for 37°C incubation.
- Plate seal or Parafilm M.
- Distilled water.
- 1X TE buffer pH 7.5 to 8.0.
- Isolated RNA of interest.

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.

If Wash Buffer contains salt precipitates, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved.

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 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 Negative Control

Ready to use as supplied.

5.4 Positive Control

Prepare in Step 6.

5.5 Detection Antibody

Dilute Detection Antibody with 1X Wash Buffer at a ratio of 1:2000 (i.e., add 1 µL of Detection Antibody to 2000 µL of 1X Wash Buffer). About 50 µL of this diluted Detection Antibody will be required for each assay well.

5.6 Capture Antibody

Dilute Capture Antibody with 1X Wash Buffer at a ratio of 1:1000 (i.e., add 1 µL of Capture Antibody to 1000 µL of 1X Wash Buffer). About 50 µL of this diluted Capture Antibody will be required for each assay well.

5.7 Enhancer Solution

Dilute Enhancer Solution with 1X Wash Buffer at a ratio of 1:5000 (i.e., add 1 µL of Enhancer Solution to 5000 µL of 1X Wash Buffer). About 50 µL of this diluted Enhancer Solution will be required for each assay well.

5.8 Fluoro Developer

Prepare just before assay (Step 8.3.1).

5.9 Fluoro Enhancer

Prepare just before assay (Step 8.3.1).

5.10 Dilution Buffer

Ready to use as supplied.

5.11 8-Well Assay Strips

Ready to use as supplied.

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

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. **For a single point Positive Control:** Dilute Positive Control with 1X TE to 0.5 ng/µL (1 µL Positive Control + 3 µL 1X TE).
2. **For a Standard Curve Positive Control:** Dilute 3 µL of Positive Control with 9 µL of 1X TE to make Diluted Positive Control (0.5 ng/µL). Mix well. Then prepare six concentration points for the control by combining Diluted Positive Control and 1X TE according to the following chart. Mix well.

Tube	Diluted Positive Control (0.5 ng/µL)	1X TE	Resulting Positive Control concentration
1	1.0 µL	49.0 µL	0.01 ng/µL
2	1.0 µL	24.0 µL	0.02 ng/µL
3	1.0 µL	9.0 µL	0.05 ng/µL
4	1.0 µL	4.0 µL	0.1 ng/µL
5	2.0 µL	3.0 µL	0.2 ng/µL
6	4.5 µL	0.0 µL	0.5 ng/µL

Δ Note Keep each of the diluted solutions on ice until use. Any remaining diluted solutions, should be discarded if not used within the same day.

7. Sample Preparation

General sample information:

Input RNA Amount:

Total RNA amount can range from 100 ng to 300 ng per reaction. An optimal amount is 200 ng per reaction. Starting RNA may be in water or in a buffer such as TE. You can use your method of choice for RNA isolation.

Storage:

Isolated total RNA can be stored at -20°C (short term) or -80°C (long term) until use.

8. Assay Procedure

– Assay all standards, controls and samples in duplicate.

8.1 RNA Binding:

1. Predetermine the number of strip-wells 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. Add 80 µL of Binding Solution to each well.
3. Add 2 µL of Negative Control, 2 µL of diluted Positive Control (from Step 6), and 200 ng of your sample RNA (1-8 µL) into the designated wells depicted in Table 1 or Table 2. Mix solution by gently tilting from side to side or shaking the plate several times. Ensure the solution coats the bottom of the well evenly.
   Δ Note For optimal binding, sample RNA volume added should not exceed 8 µL.
   Δ Note To ensure that Negative Control, diluted Positive Control and sample DNA are completely added into the wells, the pipette tip should be placed into the Binding Solution in the well and aspirated in/out 1-2 times.
4. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 minutes.
5. Remove the Binding Solution from each well. Wash each well with 150 µL of 1X Wash Buffer by pipetting 1X Wash Buffer into the wells and then removing it using a pipette. Repeat the wash two times for a total of three washes.

Well	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	NC	Sample	…	…	
B	PC	PC	Sample	…	…	
C	Sample	Sample	…	…	…	
D	…	…	…			
E						
F						
G						
H						

Table 1. The suggested strip-well plate setup using a single point positive control in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can be measured in duplicate.

Well	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	NC	Sample	…	…	
B	PC 0.02 ng/mL	PC 0.02 ng/mL	Sample	…	…	
C	PC 0.04 ng/mL	PC 0.04 ng/mL	Sample	…	…	
D	PC 0.1 ng/mL	PC 0.1 ng/mL	Sample	…		
E	PC 0.2 ng/mL	PC 0.2 ng/mL	Sample	…		
F	PC 0.4 ng/mL	PC 0.4 ng/mL	Sample			
G	PC 1 ng/mL	PC 1 ng/mL	Sample			
H	Sample	Sample	Sample			

Table 2. The suggested strip-well plate setup for standard curve preparation in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can be measured in duplicate.

8.2 m6A RNA Capture:

1. Add 50 µL of diluted Capture Antibody to each well, then cover and incubate at room temperature for 60 minutes.
2. Remove the diluted Capture Antibody solution from each well using a pipette.
3. Wash each well with 150 µL of 1X Wash Buffer each time for three times.
4. Add 50 µL of diluted Detector Antibody to each well, then cover and incubate at room temperature for 30 minutes.
5. Remove the diluted Detector Antibody solution from each well using a pipette.
6. Wash each well with 150 µL of 1X Wash Buffer each time for four times.
7. Add 50 µL of diluted Enhancer Solution to each well, then cover and incubate at room temperature for 30 minutes.
8. Remove the diluted Enhancer Solution from each well.
9. Wash each well with 150 µL of 1X Wash Buffer each time for five times.

8.3 Signal Detection:

1. Prepare Fluoro Developer Mixture by adding 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer into each 500 µL of Dilution Buffer.
2. Add 50 µL Fluoro Developer Mixture into the wells and incubate at room temperature for 1-4 minutes away from the light. The color in the standard wells containing the higher concentrations may turn pink during this period.
3. Measure and read the RFU on a fluorescence microplate reader at Ex/Em = 530/590 nm.

9. Data Analysis

Relative Quantification: To determine the relative m^{6}A RNA methylation status of two different RNA samples, a simple calculation for the percentage of m^{6}A in your total RNA can be carried out using the following formula:

$$\text{m6A\%} = \frac{(\text{Sample RFU - Negative Control RFU})/\text{S}}{(\text{PC RFU - NC RFU})/\text{P}} * 100\%$$

S is the amount of input sample RNA in ng.
P is the amount of input positive control (PC) in ng.

Example Calculation:
Average RFU of Negative Control (NC) is 900
Average RFU of Positive Control is 10900
Average RFU of sample is 1900
S is 200 ng
P is 1 ng

$$\text{m6A\%} = \frac{(1900 - 900)/200}{(10900 - 900)/1} * 100\% = 0.05\%$$

Absolute Quantification: To quantify the absolute amount of m^{6}A using an accurate calculation, first generate a standard curve and plot the RFU values (background (NC)-subtracted) versus the amount of PC at each concentration point. Next, determine the slope (RFU/ng) of the standard curve using linear regression (Microsoft Excel's linear regression functions are suitable for such calculation). Use the most linear part of the standard curve (include at least 4 concentration points) for optimal slope calculation. Now calculate the amount and percentage of m^{6}A in your total RNA using the following formulas:

$$\text{m6A(ng)} = \frac{(\text{Sample RFU - Negative Control RFU})}{\text{Slope}}$$

$$m6A\ \% = \frac{(\text{m6A amount (ng)})}{\text{Slope}} \text{ x } 100\%$$

S is the amount of input sample RNA in ng.

Example Calculation:
Average RFU of Negative Control is 900
Average RFU of sample is 1900
Slope is 10000 RFU/ng
S is 200 ng

$$\text{m6A (ng)} = \frac{(1900 - 900)}{(10000)} = 0.1 \text{ ng}$$

$$\text{m6A \%} = \frac{(0.1)}{(200)} \text{ x } 100\% = 0.05\%$$

10. Typical Data

Data provided **for demonstration purposes only**.

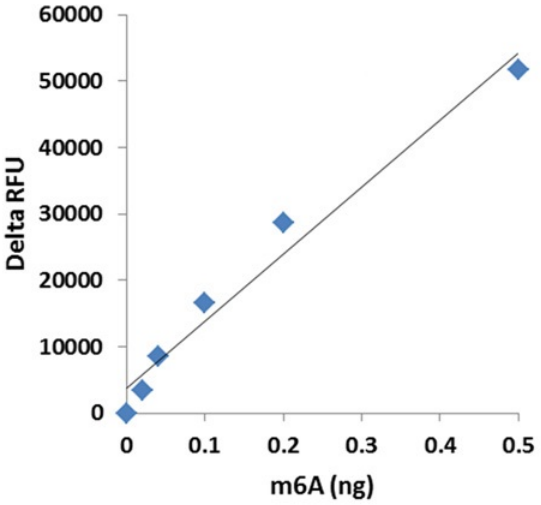


Figure 1. Example of a standard curve generated with m6A RNA Methylation Assay Kit (ab233491).

11. Troubleshooting

Problem	Reason	Solution
No signal in both the positive control and 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.
	The well is incorrectly washed before RNA binding.	Ensure the well is not washed prior to adding the positive control and sample.
	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 input materials.	Ensure that a sufficient amount of positive control (> 0.02 ng) and sample (200 ng) is added into the wells.
	Incorrect absorbance reading.	Check if appropriate absorbance wavelength (Ex/Em = 530/590 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 positive control wells	The Positive Control is insufficiently added to the well.	Ensure a sufficient amount of positive control RNA is added.
	The Positive Control is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance in this User Guide for storage of Positive Control.

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 sample or positive control.	Ensure the well is not contaminated from adding sample or positive control DNA accidentally or from using contaminated tips.
	Incubation time is too long.	The incubation time at Step 8.1.4 should not exceed 2 hours.
	Over development of fluorescence.	Decrease the development time in Step 8.3.2.
No signal or weak signal only in sample wells	RNA sample is not properly extracted or purified.	Ensure the RNA sample is good quality. The 260/280 ratio should be >1.9 with no or minimal DNA contamination.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of RNA is used as indicated in Step 8.1.3.
	Little or no m^6A contained in the sample.	N/A
Large variation between replicate wells	Fluorescent reaction is not evenly occurring due to an inconsistency in pipetting time	Ensure e Fluoro-Development Solution is added at the same time between replicates or otherwise maintain a consistent timing in between each addition of solutions.
	Fluorescent reaction is not occurring evenly due to an inconsistent order of adding solutions.	Ensure all solutions, particularly Fluoro Development Mixture, are added in the same order each time as all other solutions.

Large variation between replicate wells	The solutions are not evenly added due to an 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 volumes (e.g., 1 µL) 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
	Did not sufficiently shake the solutions in the wells after adding sample or positive control at Step 8.1.3.	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

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