ab185912 – m6A RNA Methylation Quantification Kit (Colorimetric)

Instructions for Use

For the quantification of m6A RNA Methylation in various samples

View kit datasheet: www.abcam.com/ab185912
(use www.abcam.cn/ab185912 for China, or www.abcam.co.jp/ab185912 for Japan)

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

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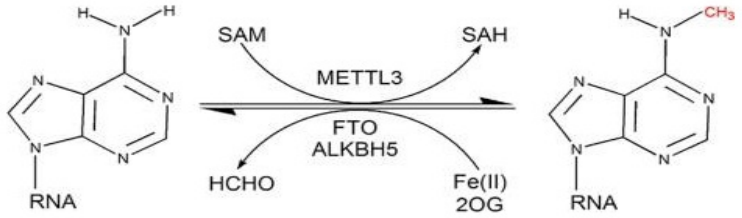
1. BACKGROUND

N6-methyladenosine (m6A) is the most common and abundant modification in RNA molecules present in eukaryotes. The m6A modification is catalyzed by a methyltransferase complex METTL3 and removed by the recently discovered m6A RNA demethylases FTO and ALKBH5, which catalyze m6A demethylation in an α-ketoglutarate (α-KG)- and Fe2+-dependent manner. It was shown that METTL3, FTO, and ALKBH5 play important roles in many biological processes, ranging from development and metabolism to fertility. m6A accounts for more than 80% of all RNA base methylations and exists in various species. m6A is mainly distributed in mRNA and also occurs in non-coding RNA such as tRNA, rRNA, and snRNA. The relative abundance of m6A in mRNA transcripts has been shown to affect RNA metabolism processes such as splicing, nuclear export, translation ability and stability, and RNA transcription. Abnormal m6A methylation levels induced by defects in m6A RNA methylase and demethylase could lead to dysfunction of RNA and diseases. For example, abnormally low levels of m6A in target mRNAs due to increased FTO activity in patients with FTO mutations, through an as-yet undefined pathway, contributes to the onset of obesity and related diseases. The dynamic and reversible chemical m6A modification in RNA may also serve as a novel epigenetic marker of profound biological significance. Therefore, more useful information for a better understanding of m6A RNA methylation levels and distribution on RNA transcripts could benefit diagnostics and therapeutics of disease.



Several chromatography-based techniques such as HPLC-ECD and LC-MS are used for detecting m6A in tissues and cells. However these methods are time consuming and have low throughput with high costs. To address this issue, Abcam offers the m6A RNA Methylation Quantification Kit (Colorimetric) (ab185912).

The kit has the following advantages and features:

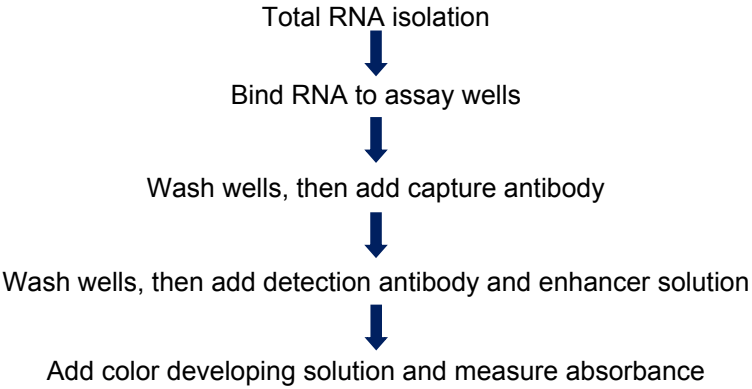
- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 3 hours and 45 minutes.
- High sensitivity, of which the detection limit can be as low as 10 pg of m6A.
- Unique binding solution allows RNA >70 nts to be tightly bound to the wells, which enables quantification of m6A from both mRNA and ncRNA such as tRNA, rRNA, and snRNA.
- Optimized antibody and enhancer solutions allow high specificity to m6A, with no cross-reactivity to unmethylated adenosine within the indicated concentration range of the sample RNA.
- Universal positive and negative controls are included, which are suitable for quantifying m6A from any species.
- Strip microplate format makes the assay flexible: manual or high throughput analysis (96 assays)

INTRODUCTION

The m6A RNA Methylation Quantification Kit (Colorimetric) (ab185912) is suitable detecting N6-methyladenosine (m6A) RNA methylation status directly using total RNA isolated from any species such as mammals, plants, fungi, bacteria, and viruses.

In this assay, total RNA is bound to strip wells using a RNA high binding solution. N6-methyladenosine (m6A) is detected using a specific capture N6-methyladenosine antibody and detection antibody. The detected signal is enhanced and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The amount of m6A is proportional to the OD intensity measured. Both negative and positive RNA controls are provided in this kit. A standard curve can be performed (range: 0.02 to 1 ng of m6A) or a single quantity of m6A can be used as a positive control. Because m6A content can vary from tissue to tissue, and from normal and diseased states, or vary under treated and untreated conditions, it is advised to run replicate samples to ensure that the signal generated is validated. This kit will allow the user to quantify an absolute amount of m6A and determine the relative m6A RNA methylation states of two different RNA samples.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt.

Observe the storage conditions for individual prepared components in sections 9 & 10.

For maximum recovery of the products, centrifuge the original vial prior to opening the cap.

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

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
Binding Solution	5 mL	10 mL	RT
Negative Control	10 µL	20 µL	–20°C
Positive Control	10 µL	20 µL	–20°C
Capture Antibody	5 µL	10 µL	4°C
Detection Antibody	6 µL	12 µL	–20°C
Enhancer Solution	5 µL	10 µL	–20°C
Developer Solution	5 mL	10 mL	4°C
Stop Solution	5 mL	10 mL	RT
8-Well Assay Strips (With Frame)	6	12	4°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Microplate reader capable of reading absorbance at 450 nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Isolated RNA of interest
- 1X TE buffer pH 7.5 to 8.0
- Parafilm M or aluminum foil

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

9. REAGENT PREPARATION

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

9.1 1X Wash Buffer

Add the volume specified in the table below of 10X Wash Buffer to distilled water and adjust to pH 7.2-7.5.

	Volume to Dilute (mL)	Volume distilled water (mL)	Total Volume (mL)
48 Tests	13	117	130
96 Tests	26	234	260

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

9.2 Capture Antibody Solution

Dilute Capture Antibody with Diluted 1X Wash Buffer at a ratio of 1:1000 (i.e., 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.

9.3 Detection Antibody Solution

Dilute Detection Antibody with Diluted 1X Wash Buffer at a ratio of 1:2000 (i.e., 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.

9.4 Enhancer Solution

Dilute Enhancer Solution Enhancer Solution with Diluted 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 Diluted Enhancer Solution will be required for each assay well.

10. STANDARD PREPARATION

10.1 Single Point Positive Control

Dilute Positive Control with 1X TE Buffer to 0.5ng/µL (i.e., add 1 µL of Positive Control to 3 µL of TE Buffer).

10.2 Standard Curve

First, dilute Positive Control to 0.5 ng/µL (ex: 3 µL of Positive Control + 9 µL of 1X TE). Then, further prepare 6 different concentrations with the 0.5 ng/ µL Positive Control and 1X TE into 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 ng/ µL according to the following dilution chart.

Tube	Diluted Positive Control (µL)	1X TE (µL)	Final Concentration (ng/ µL)
1	1.0	49	0.01
2	1.0	24	0.02
3	1.0	9	0.05
4	1.0	4	0.10
5	2.0	3	0.20
6	4.5	0	0.50

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

11. SAMPLE PREPARATION

Starting Materials: Starting materials can include various tissue or cell samples such as cells from flask or microplate cultured cells, fresh and frozen tissues, paraffin-embedded tissues, blood, body fluid samples, etc.

Input RNA Amount: Total RNA amount can be 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.

RNA Storage: Isolated total RNA should be stored in aliquots at –20°C or –80°C until use.

12. ASSAY PROCEDURE

Internal Control: Because RNA Methylation can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated.

12.1 **RNA Binding**

12.1.1 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).

12.1.2 Add 80 µL of Binding Solution to each well.

12.1.3 Add 2 µL of Negative Control, 2 µL of Diluted Positive Control and 200 ng of your sample RNA (1-8 µL) into the designated wells (see the designated wells depicted in Table 1 or Table 2 below). 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.

Note: *For a single point control, add 2 µL of Positive Control at concentrations of 0.5 ng/ µL. For the standard curve, add 2 µL of Diluted Positive Control at concentrations of 0.01 to 0.5 ng/ µL (see Standard Preparation Section). The final concentrations should be 0.02, 0.2, 0.1, 0.4, and 1 ng per well. For optimal binding, sample RNA volume added should not exceed 8 µL. 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.*

12.1.4 Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 min.

12.1.5 Remove the Binding Solution from each well. Wash each well three times with 150 µL of the Diluted 1X Wash Buffer each time.

12.2 **m6a RNA Capture**

12.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then cover Parafilm M or aluminium foil and incubate at room temperature for 60 min.

12.2.2 Remove the Diluted Capture Antibody solution from each well.

12.2.3 Wash each well three times with 150 µL of the Diluted 1X Wash Buffer each time.

12.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

12.2.5 Remove the Diluted Detection Antibody solution from each well.

12.2.6 Wash each well four times with 150 µL of the Diluted 1X Wash Buffer each time.

12.2.7 Add 50 µL of the Diluted Enhancer Solution to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 min.

12.2.8 Remove the Diluted Enhancer Solution from each well.

12.2.9 Wash each well five times with 150 µL of the Diluted 1X Wash Buffer each time.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed at each wash step. The wash can be carried out by simply pipetting the wash buffer into the wells and then pipetting the buffer out from the wells (discard the buffer).*

12.3 **Signal Detection**

12.3.1 Add 100 µL of Developer Solution to each well and incubate at room temperature for 1 to 10 min away from light. Begin monitoring color changes in the sample wells and control wells. The Developer Solution will turn blue in the presence of sufficient m6A.

12.3.2 Add 100 µL of Stop Solution to each well to stop enzyme reaction when the color in the positive control wells turns medium blue. The color will change to yellow after adding Stop Solution and the absorbance should be read on a microplate reader within 2 to 10 min at 450 nm.

Note: *If the stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

Well	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip6
A	Negative Control	Negative Control	Sample	Sample	Sample	Sample
B	Positive Control	Positive Control	Sample	Sample	Sample	Sample
C	Sample	Sample	Sample	Sample	Sample	Sample
D	Sample	Sample	Sample	Sample	Sample	Sample
E	Sample	Sample	Sample	Sample	Sample	Sample
F	Sample	Sample	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

Table 1. The suggested strip-well plate setup for 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	Strip6
A	Negative Control	Negative Control	Sample	Sample	Sample	Sample
B	Positive Control 0.02 ng/well	Positive Control 0.02 ng/well	Sample	Sample	Sample	Sample
C	Positive Control 0.04 ng/well	Positive Control 0.04 ng/well	Sample	Sample	Sample	Sample
D	Positive Control 0.1 ng/well	Positive Control 0.1 ng/well	Sample	Sample	Sample	Sample
E	Positive Control 0.2 ng/well	Positive Control 0.2 ng/well	Sample	Sample	Sample	Sample
F	Positive Control 0.4 ng/well	Positive Control 0.4 ng/well	Sample	Sample	Sample	Sample
G	Positive Control 1 ng/well	Positive Control 1 ng/well	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	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.

13. ANALYSIS

m6A Calculation

Relative Quantification:

To determine the relative m6A RNA methylation status of two different RNA samples, a simple calculation for the percentage of m6A in your total RNA can be carried out using the following formula:

$$m6A\% = \frac{(Sample\ OD - NC\ OD) \div S}{(PC\ OD - NC\ OD) \div P} \times 100\%$$

S is the amount of input sample RNA in ng.
P is the amount of input Positive Control in ng.

Example calculation:

Average OD450 of Negative Control (NC) is 0.1

Average OD450 of Positive Control(PC) is 0.4

Average OD450 of sample is 0.16

S is 200 ng

P is 1 ng

$$m6A = \frac{(0.16 - 0.1) \div 200}{(0.4 - 0.1) \div 1} \times 100\% = 0.1\%$$

Absolute Quantification:

First, generate a standard curve and plot the OD values (background (Negative Control)-subtracted) versus the amount of Positive Control at each concentration point. Then determine the slope as OD/ng using linear regression (Microsoft Excel's linear regression or slope 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 percentage of m6A in your total RNA using the following formulae:

$$m6A\ \% = \frac{m6A\ Amount\ (ng)}{S} \times 100\%$$

S is the amount of input sample RNA in ng.
NC is OD if Negative Control

Example calculation:

Average OD450 of Negative Control is 0.1
Average OD450 of sample is 0.16
Slope is 0.3 OD/ng
S is 200 ng

$$m6A\ = \frac{0.16 - 0.1}{0.3}\ = 0.2\ ng$$

$$m6A\ \% = \frac{0.2}{200} \times\ 100\%\ = 0.1\ \%$$

Typical Results

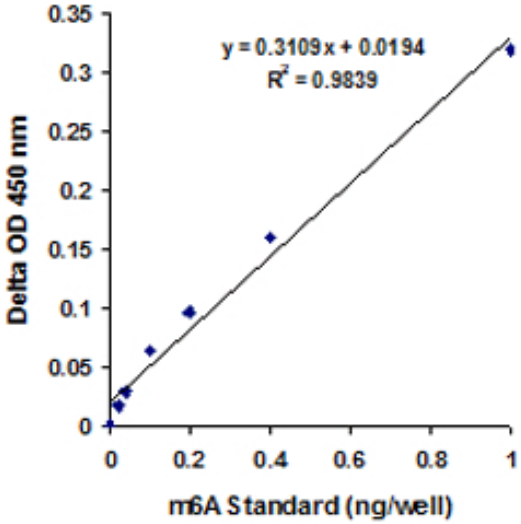


Fig. 1. m6A standard control was added into the assay wells at different concentrations and then measured with the m6A RNA Methylation Quantification Kit (Colorimetric) (ab185912).

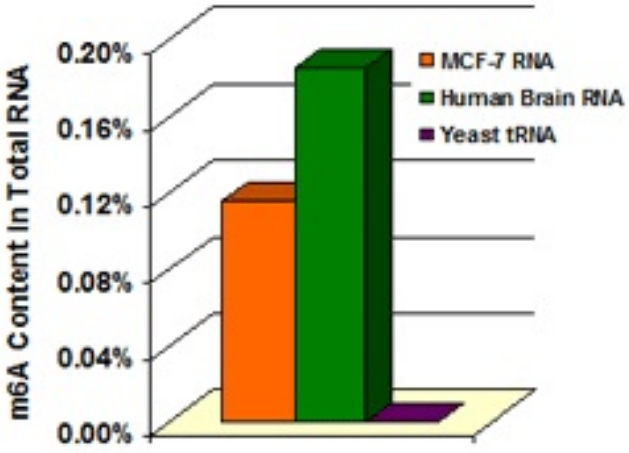


Fig. 2 Quantification of m6A RNA methylation in different samples. 200 ng of RNA isolated from different tissues or cells were added into the assay wells and the m6A contained in RNA was measured using the m6A RNA Methylation Quantification Kit (Colorimetric) (ab185912).

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14. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the standard and sample wells	Reagents are added incorrectly	Check if reagents are added in the proper order with the right amount, 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 incubation time and temperature described in the protocol is followed correctly
	Insufficient input materials	Ensure that a sufficient amount of positive control (> 0.2 ng) and sample (200 ng) is added into the wells
	Incorrect absorbance reading	Check if the 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 cap is tightly secured after each opening or use

No signal or weak signal in only the Positive control wells	The Positive Control amount is insufficiently added to the well	Ensure a sufficient amount of Positive Control is added
	The Positive Control is degraded due to improper storage conditions	Follow the Shipping & Storage guidelines of this User Guide for storage of PC (Positive Control)
High background present in the blank wells	Insufficient washing of wells	Check if washing at each step is performed according to the protocol
	Contaminated by sample or standard	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips
	Incubation time is too long	The incubation time at should not exceed 45 minutes
	Over development of color	Decrease the development time before adding Stop Solution
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

Uneven color development	Insufficient wash of the wells	Ensure the wells are washed according to the guidance of washing and residue washing buffer is removed as much as possible
	Delayed color development or delayed stopping of color development in the wells	Ensure color development and stop solutions are added sequentially and consistent with the order you added the other reagents (e.g., from well A to G or from well 1 to 12)

15. NOTES

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