Ab185912 – m6A RNA Methylation Quantification Kit (Colorimetric)

For the quantification of m6A RNA Methylation in various samples. This product is for research use only and is not intended for diagnostic use.
For overview, typical data and additional information please visit: www.abcam.com/ab185912 (use abcam.cn/ab185912 for China or scan QR code via WeChat, or abcam.co.jp/ab185912 for Japan)

Materials Supplied and Storage

Store kit as given in the table upon receipt.
Observe the storage conditions for individual prepared components.
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

Item	48 Tests	96 Tests	Storage Condition (Before Prep.)
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

Materials Required, Not Supplied

- 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

1. Reagent Preparation

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

1.1 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. The 1X Wash Buffer can be stored at 4°C for up to 6 months.

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

1.2 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.

1.3 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.

1.4 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.

2. Standard Preparation

2.1 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).
2.2 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.

3. 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.

4. Assay Procedure

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

4.1 RNA Binding

4.1.1 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).

4.1.2 Add 80 µL of Binding Solution to each well.

4.1.3 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 RNA 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.*

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

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

4.2 m6a RNA Capture

4.2.1 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.

4.2.2 Remove the Diluted Capture Antibody solution from each well.

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

4.2.4 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.

4.2.5 Remove the Diluted Detection Antibody solution from each well.

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

4.2.7 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.

4.2.8 Remove the Diluted Enhancer Solution from each well.

4.2.9 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).*

4.3 Signal Detection

4.3.1 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.

4.3.2 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 within 2 to 10 min at 450 nm.

Note: *If the strip well 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	Neg Control	Neg Control	Sample	Sample	Sample	Sample
B	Pos Control 0.02 ng/well	Pos Control 0.02 ng/well	Sample	Sample	Sample	Sample
C	Pos Control 0.04 ng/well	Pos Control 0.04 ng/well	Sample	Sample	Sample	Sample
D	Pos Control 0.1 ng/well	Pos Control 0.1 ng/well	Sample	Sample	Sample	Sample
E	Pos Control 0.2 ng/well	Pos Control 0.2 ng/well	Sample	Sample	Sample	Sample
F	Pos Control 0.4 ng/well	Pos Control 0.4 ng/well	Sample	Sample	Sample	Sample
G	Pos Control 1 ng/well	Pos 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.

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:

$$m6A\ (ng) = \frac{Sample\ OD - NC\ OD}{Slope}$$

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

S is the amount of input sample RNA in ng.

NC is 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

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

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

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