ab326115 – m6A Methylase Assay Kit (Colorimetric)

For overview, typical data and additional information please visit: www.abcam.com/ab326115

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The m6A Methylase Activity/Inhibition Assay Kit (Colorimetric) is suitable for measuring the activity/inhibition of total m6A methylases (methyltransferases) using nuclear extracts or purified m6A methylases like METTL3/METTL14 from a broad range of species such as mammalian, plant, fungal, and bacterial, in a variety of forms including, but not limited to, cultured cells and, fresh and frozen tissues.

Starting Materials: Input materials can be nuclear extracts or purified enzymes. The amount of nuclear extracts for each assay can be 2 µg to 20 µg with an optimal range of 5 µg to 10 µg. The amount of purified m6A RNA methylases can be 20 ng to 1 µg with an optimal range of 50 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

Internal Control: An assay standard is provided in this kit for the quantification of m6A methylase activity. Because m6A methylase activity 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.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

KIT CONTENTS

Component	48 Assays	96 Assays	Storage Upon Receipt
WB (10X Wash Buffer)	14 ml	28 ml	+4°C
MB (Methylase Buffer)	3 ml	6 ml	RT
MMS (m6A Methylase Substrate)*	100 µl	200 µl	- 20°C
SAM (S-adenosylmethionine, 400X)*	20 µl	40 µl	- 20°C
BS (Binding Solution)	5 ml	10 ml	RT
AS (Assay Standard, 2 µg/ml)*	10 µl	20 µl	- 20°C
CA (Capture Antibody, 1000 µg/ml)*	5 µl	10 µl	+4°C
DA (Detection Antibody, 400 µg/ml)*	6 µl	12 µl	- 20°C
ES (Enhancer Solution)*	5 µl	10 µl	- 20°C
DS (Developer Solution)	5 ml	10 ml	+4°C
SS (Stop Solution)	5 ml	10 ml	RT
8-Well Assay Strips (With Frame)	6	12	+4°C

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

SHIPPING & STORAGE

The kit is shipped in three parts: the first part at ambient room temperature, and the second and third parts on frozen ice packs at 4°C. Upon receipt: (1) store **MMS**, **SAM**, **AS**, **DA**, and **ES** at –20°C away from light; (2) store **WB**, **CA**, **DS**, and **8-Well Assay Strips** at 4°C away from light; (3) store remaining components (**MB**, **BS** and **SS**) at room temperature away from light.

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

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

MATERIALS REQUIRED BUT 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 and 655 nm
- 1.5 ml microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract or purified enzymes
- Parafilm M or aluminum foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of m6A Methylase Activity/Inhibition Assay Kit (Colorimetric) is tested against predetermined specifications to ensure consistent product quality. Abcam guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply contact our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: Abcam reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice.

Usage Limitation: The m6A Methylase Activity/Inhibition Assay Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application.

Intellectual Property: The m6A Methylase Activity/Inhibition Assay Kit (Colorimetric) and methods of use contain proprietary technologies by Abcam.

ASSAY PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

***Input Amount*:** The amount of nuclear extracts for each assay can be 2 µg to 20 µg with an optimal range of 5 µg to 10 µg. The amount of purified enzymes can be 20 ng to 1 µg with an optimal range of 50 ng to 500 ng, depending on the purity and catalytic activity of the enzymes.

***Nuclear Extraction*:** You can use your method of choice for preparing nuclear extracts. Abcam offers a nuclear extraction kit optimized for use with this kit.

***Nuclear Extract or Purified Enzyme Storage*:** Nuclear extract or purified enzyme should be stored in aliquots at –80°C until use.

1. **Buffer Solution & Preparation**

a. Prepare **Diluted WB** (1X Wash Buffer):
48-Assay Kit: Add 13 ml of **WB** (10X Wash Buffer) to 117 ml of distilled water and adjust pH to 7.2-7.5.
96-Assay Kit: Add 26 ml of **WB** (10X Wash Buffer) to 234 ml of distilled water and adjust pH to 7.2-7.5.
This **Diluted WB** (1X Wash Buffer) can now be stored at 4°C for up to six months.

b. Prepare **Working MB**:
Add **SAM** (S-adenosylmethionine) to **MB** (Methylase Buffer) at a ratio of 1:400 (i.e., add 1 µl of each **SAM** to 399 µl of **MB** for a total of 400 µl). About 50 µl of **Working MB** will be required for each assay well.

c. Prepare **Diluted CA**:
Dilute **CA** (Capture Antibody) with **Diluted WB** (1X Wash Buffer) at a ratio of 1:1000 (i.e., add 1 µl of **CA** to 1000 µl of **Diluted WB** (1X Wash Buffer). About 50 µl of **Diluted CA** will be required for each assay well.

d. Prepare **Diluted AS**:
Suggested Standard Curve Preparation: First, dilute **AS** (Assay Standard) with **MB** to 1 ng/µl by adding 5 µl of **AS** to 5 µl of **MB**. Then, further prepare five concentrations by combining the 1 ng/µl **Diluted AS** with **MB** into final concentrations of 0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 ng/µl according to the following dilution chart:

Tube	Diluted AS (1 ng/µl)	MB	Resulting Diluted AS Concentration
1	1.0 µl	39.0 µl	0.02 ng/µl
2	1.0 µl	19.0 µl	0.05 ng/µl
3	1.0 µl	9.0 µl	0.1 ng/µl
4	1.0 µl	4.0 µl	0.2 ng/µl
5	2.0 µl	2.0 µl	0.5 ng/µl
6	4.0 µl	0.0 µl	1.0 ng/µl

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

2. Enzymatic Reaction

a. 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).
b. Add 80 µl of **BS** (Binding Solution) to each well.
c. Add 2 µl of **MMS** (m6A Methylase Substrate) into each sample well. Add 2 µl of **Working MB** into blank well. Add 1 µl of **Diluted AS** into the standard curve wells (see the designated wells depicted in Table 2 under "Suggested Strip Well Setup" 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 the standard curve, add 1 µl of* ***Diluted AS*** *at concentrations of 0.02 to 1 ng/µl (see the chart in Step 1g). The final concentrations should be 0.02, 0.05, 0.1, 0.2, 0.5, and 1 ng per well.*
d. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 min.
e. Remove the **BS** (Binding Solution) from each well.
f. Wash each well two times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.
g. Blank Wells: Add 50 µl of **Working MB** to each blank well.
h. Standard Wells: Add 50 µl of **Working MB** to each standard well.
i. Sample Wells Without Inhibitor: Add 46 to 49 µl of **Working MB** and 1 to 4 µl of nuclear extracts or purified enzyme to each sample well without inhibitor. Total volume should be 50 µl per well.
j. Sample Wells With Inhibitor: Add 41 to 44 µl of **Working MB**, 1 to 4 µl of nuclear extracts or purified enzyme, and 5 µl of inhibitor solution. Total volume should be 50 µl per well.
Note*: (1) Follow the suggested well setup diagrams under "Suggested Strip Well Setup"; (2) It is recommended to use 5 µg to 10 µg of nuclear extract per well or 50 ng to 500 ng of purified enzyme per well; (3) The concentration of inhibitor to be added into the sample wells can be varied (1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with* ***MB*** *at a 1:10 ratio (i.e., add 0.5 µl of inhibitor to 4.5 µl of* ***MB****) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*
k. Tightly cover strip plate with **Parafilm M** to avoid evaporation and incubate at 37°C for 60-90 min.
∆***Note****: The incubation time may depend on intrinsic enzyme activity. However, in general, 60 min incubation is suitable for active purified m6A methylase enzyme and 90 min incubation is required for nuclear extract.*
l. Remove the reaction solution from each well. Wash each well three times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.

3. Antibody Binding & Signal Enhancing

a. Add 50 µl of the **Diluted CA** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.
b. During the last 10 minutes of Diluted CA incubation, prepare the **Immuno-Detection Complex Solution**: In each 1 ml of **Diluted WB** (1 X wash buffer) add 0.5 µl of **DA** (Detection Antibody) and then add 0.5 µl of **ES** (Enhancer Solution). Mix well.
c. Remove the **Diluted CA** solution from each well.
d. Wash each well two times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.
e. Add 50 µl of the **Immuno-Detection Complex** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.
f. Remove the **Immuno-Detection Complex** solution from each well.
g. Wash each well four times with 150 µl of the **Diluted WB** (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. Signal Detection

a. Add 100 µl of **DS** (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 **DS** (Developer Solution) will turn blue in the presence of sufficient methylated m6A.
b. Add 100 µl of **SS** (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 **SS** (Stop solution) and the absorbance should be read on a microplate reader within 2 to 10 min at 450 nm with an optional reference wavelength of 655 nm.
∆***Note**: (1) Most microplate readers have capability to carry out dual wavelength analysis and will automatically subtract reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, the plate can be read twice – once at 450 nm and once at 655 nm. Then manually subtract the 655 nm ODs from 450 nm ODs; (2) If the stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

5. m6A Methylase Activity Calculation

a. Calculate the average for duplicate readings for sample wells and blank wells.
b. Calculate enzyme activity or inhibition using the following formulas:

For simple calculation:

$$\text{Methylase activity (OD/h/mg)} = \frac{\text{OD (sample – blank)}}{\text{[Protein Amount (µg)/1000]* X Hour **}}$$

* Protein amount added into the reaction at Step 2i or 2j.
** Incubation time at Step 2k (in hours).
Example calculation:
Average OD450 of sample is 0.65
Average OD450 of blank is 0.05
Protein amount is 5 µg
Incubation time is 1 hour (60 min)

$$\text{Activity} = \frac{(0.35 - 0.05)}{(5/1000 \times 1)} = 60 \text{ OD/h/mg}$$

For accurate or specific activity calculation:
First, generate a standard curve and plot the OD values versus the amount of **AS** (Assay Standard) 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 m6A methylase activity using the following formula:

$$\text{Activity (ng/h/mg)} = \frac{\text{OD (sample – blank)}}{\text{Slope} \times \text{Protein Amount (µg)* X Hour **}} \times 1000$$

* Protein amount added into the reaction at Step 2i or 2j
** Incubation time at Step 2k (in hours).
For inhibition calculation:

$$\text{Inhibition \%} = \left(1 - \frac{\text{OD (inhibitor sample – blank)}}{\text{OD (no inhibitor sample – blank)}}\right) \times 100\%$$

SUGGESTED STRIP WELL SETUP

Table 1. Approximate amount of required buffers and solutions for defined assay wells based on the protocol.

Reagents	1 well	1 strip (8 wells)	2 strips (16 wells)	6 strips (48 wells)	12 strips (96 wells)
Diluted WB	2.5 ml	20 ml	40 ml	120 ml	240 ml
MB	50 µl	400 µl	800 µl	2400 µl	4800 µl
MS	2 µl	16 µl	32 µl	96 µl	192 µl
SAM	0.2 µl	1 µl	2 µl	6 µl	12 µl
AS	N/A	N/A	4 µl (optional)	8 µl	16 µl
BS	80 µl	650 µl	1350 µl	2700 µl	5400 µl
Diluted CA	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted DA	50 µl	400 µl	800 µl	2400 µl	4800 µl
Diluted ES	50 µl	400 µl	800 µl	2400 µl	4800 µl
DS	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
SS	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml

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 should be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	AS 0.02 ng/µl	AS 0.02 ng/µl	Sample	Sample	Sample	Sample
C	AS 0.05 ng/µl	AS 0.05 ng/µl	Sample	Sample	Sample	Sample
D	AS 0.1 ng/µl	AS 0.1 ng/µl	Sample	Sample	Sample	Sample
E	AS 0.2 ng/µl	AS 0.2 ng/µl	Sample	Sample	Sample	Sample
F	AS 0.5 ng/µl	AS 0.5 ng/µl	Sample	Sample	Sample	Sample
G	AS 1 ng/µl	AS 1 ng/µl	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

TROUBLESHOOTING

Problem	Possible Cause	Suggestion
No signal or weak signal	Reagents are added incorrectly.	Check if reagents are added in the proper order with the right amount, and

in both the standard and sample wells		if any steps in the protocol may have been omitted by mistake.
	The substrate and standard are not properly bound to the wells.	Ensure that (1) the **MS** and **AS** are added into the wells; (2) the wells are completely covered with sufficient **BS** (Binding Solution); and (3) binding time is sufficient (90 min).
	Incubation time and temperature are incorrect.	Ensure that the incubation time and temperature described in the protocol are followed correctly.
	Incorrect absorbance reading.	Check if the appropriate absorbance wavelength (450 nm filter) is used.
	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperatures and the cap is tightly secure after each opening or use.
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance of this User Guide for storage of **AS** (Assay Standard).
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 with detection antibody is too long.	The incubation time at Step 3e should not exceed 45 minutes.
	Over development of color.	Decrease the development time in Step 4a before adding **SS** (Stop Solution) in Step 4b.
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for DNA demethylase extraction. For the best results, it is advised to use Nuclear Extraction Kit. Also, use fresh cells or tissues for protein extraction, as frozen cells or tissues could lose enzyme activity.
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of nuclear extracts is used as indicated in Steps 2i and 2j. The sample can be titrated to determine the optimal amount to use in the assay.
	Sample was not stored properly or has been stored for too long.	Ensure sample is stored in aliquots at –80°C, with no more than 6 weeks for nuclear extracts. Avoid repeated freezing/thawing.
	Little or no activity of m6A methylase contained in the sample.	This problem may be a result of many factors. If the affecting factors cannot be determined, use new or re-prepared nuclear extracts.
Uneven color development	Insufficient washing of the wells.	Ensure the wells are washed according to the user guide. Ensure residual wash 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 H or from well 1 to 12).

Technical Support

Version 1 | 2026-04-22

For all technical or commercial enquiries please go to:
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