Version 1 Last updated 11 April 2018

ab233489 m6A Demethylase Assay Kit

For the measurement of the activity/inhibition of total m6A demethylases in cell/tissue nuclear extracts and purified proteins from a broad range of sources.

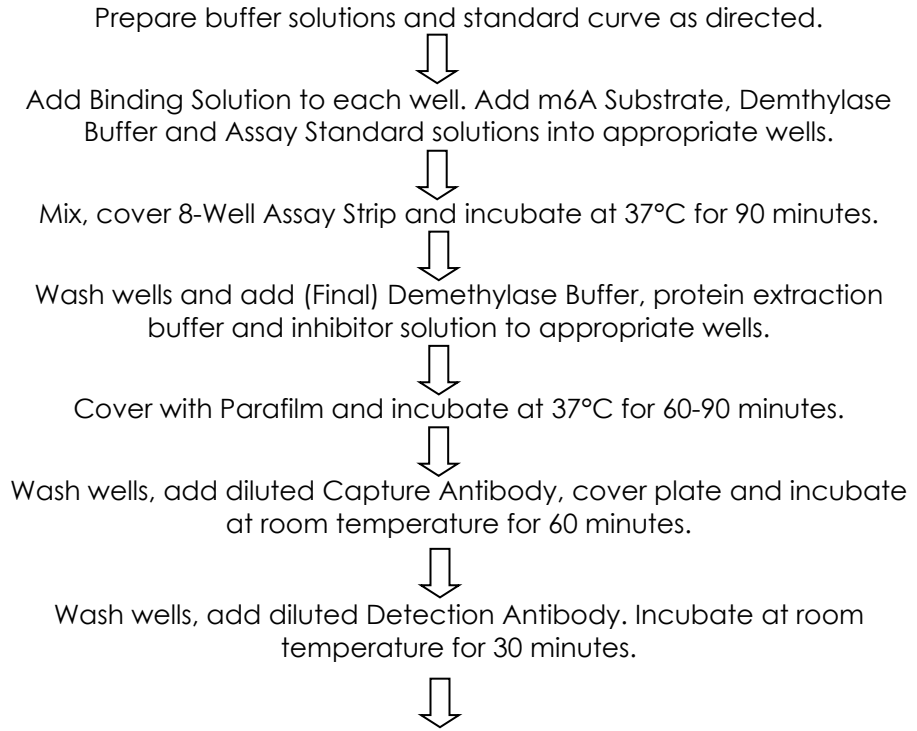
This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview

m6A Demethylase Assay Kit (ab233489) is a complete set of optimized buffers and reagents to colorimetrically measure the activity/inhibition of total m6A demethylases using nuclear extracts or purified m6A demethylases like FTO and ALKBH5. m6A demethylase activity may be measured in 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.

A unique m6A substrate is stably coated on the strip wells. Active m6A demethylases bind to and demethylate m 6A contained in the substrate. The un-demethylated m6A in the substrate can be recognized with a high affinity m6A antibody and the immuno-signal is enhanced with enhancer solution. The ratio or amount of undemethylated m6A, which is inversely proportional to enzyme activity, can then be colorimetrically quantified through an ELISA-like reaction.



Wash wells, add diluted Enhancer Solution. Incubate at room temperature for 30 minutes.

Wash wells, add Detection Solution. Incubate at room temperature for 1 to 10 minutes away from light. Monitor color changes.

Add Stop Solution to stop enzyme reaction. Read absorbance within 2 to 10 minutes at 450 nm.

2. Materials Supplied and Storage

Store 10 X m6A Substrate, Assay Standard, Detection Antibody, Enhancer Solution, Co-Factor 1, Co-Factor 2 and Co-Factor 3 at –20°C away from light. Store 10X Wash Buffer, Capture Antibody, Developer Solution, and 8-Well Assay Strips at 4°C away from light. Store Demethylase Buffer, Binding Solution and Stop Solution at room temperature away from light. Kit can be stored for 6 months 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	Quantity (48 assays)	Quantity (96 assays)	Storage temperature (before prep)
10X Wash Buffer	14 ml	28 ml	4°C
Demethylase Buffer	3 ml	6 ml	RT
10X m6A Substrate	10 µl	20 µl	-20°C
Binding Solution	5 ml	10 ml	RT
Assay Standard, 2 µg/ml	10 µl	20 µl	-20°C
Capture Antibody, 1000 µg/ml	5 µl	10 µl	4°C
Detection Antibody, 400 µg/ml	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
Co-Factor 1	30 µl	60 µl	-20°C
Co-Factor 2	30 µl	60 µl	-20°C
Co-Factor 3	30 µl	60 µl	-20°C
8-Well Assay Strips (With Frame)	6	12	4°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform 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.
- Nuclear extract or purified enzymes.
- Parafilm M or aluminium foil.

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 Co-factor 1

Ready to use as supplied

5.3 Co-factor 2

Ready to use as supplied.

5.4 Co-factor 3

Ready to use as supplied.

5.5 Demethylase Buffer

Add Co-Factor 1, Co-Factor 2 and Co-Factor 3 to Demethylase Buffer at a ratio of 1:100 (i.e., add 1 µl of each Co-Factor to 100 µl of Demethylase Buffer for a total of 103 µl). About 50 µl of this Final Demethylase Buffer will be required for each assay well.

5.6 10X m6A Substrate

Add 1 µl of 10X m6A Substrate to 9 µl of Demethylase Buffer. About 2 µl of 1X m6A Substrate will be required for each assay well.

5.7 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 diluted Capture Antibody will be required for each assay well.

5.8 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 diluted Detection Antibody will be required for each assay well.

5.9 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 diluted Enhancer Solution will be required for each assay well.

5.10 Binding Solution

Ready to use as supplied.

5.11 Developer Solution

Ready to use as supplied

.

5.12 Stop Solution

Ready to use as supplied.

5.13 8-Well Assay Strips

Ready to use as supplied.

5.14 Assay Standard

See standard preparation (Section 6).

ΔNote: Keep each of diluted solutions (except diluted 1X Wash Buffer) on ice until use. Any remaining diluted solutions other than 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. Dilute Assay Standard with Demethylase Buffer to 1 ng/µl by adding 5 µl of Assay Standard to 5 µl of Demethylase Buffer.
2. Further prepare five concentrations by combining the 1 ng/µl diluted Assay Standard with Demethylase Buffer 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 Assay Standard (1 ng/µL)	Demethylase Buffer (µL)	Resulting Diluted Assay Standard Concetration
1	1 µl	39 µl	0.02 ng/ µl
2	1 µl	19 µl	0.05 ng/ µl
3	1 µl	9 µl	0.1 ng/ µl
4	1 µl	4 µl	0.2 ng/ µl
5	2 µl	2 µl	0.5 ng/ µl
6	4 µl	0 µl	1 ng/ µl

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

7. Sample Preparation

General sample information:

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 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 series of Nuclear extraction kits for your convenience.

Nuclear Extract or Purified Enzyme Storage:

Nuclear extract or purified enzyme should be stored in aliquots at –80°C until use.

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 Enzymatic Reaction

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 1X m6A Substrate into each sample well. Add 2 µl of Demethylase Buffer into blank well. Add 1 µl of diluted Assay Standard into the standard curve wells. 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.
4. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 min.
5. Remove the Binding Solution from each well.
6. Wash each well three times with 150 µl of the diluted 1X Wash Buffer each time.
7. Blank Wells: Add 50 µl of Final Demethylase Buffer to each blank well.
8. Standard Wells: Add 50 µl of Final Demethylase Buffer to each standard well.
9. Control Wells without Nuclear Extracts: Add 45-48 µl of Demethylase Buffer and 2-5 µl of your protein extraction buffer.
10. Sample Wells Without Inhibitor: Add 46 to 49 µl of Final Demethylase Buffer 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.
11. Sample Wells With Inhibitor: Add 41 to 44 µl of Final Demethylase Buffer, 1 to 4 µl of nuclear extracts or purified enzyme, and 5 µl of inhibitor solution. Total volume should be 50 µl per well.

ΔNote: Follow the suggested well setup diagrams as indicated below in Table 1.
ΔNote: 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 Demethylase Buffer at a 1:10 ratio (i.e., add 0.5 µl of inhibitor to 4.5 µl of Demetylase Buffer) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.

12. Tightly cover strip plate with Parafilm M to avoid evaporation and incubate at 37°C for 60-90 min.

ΔNote: The incubation time may depend on intrinsic enzyme activity. However, in general, 60 min incubation is suitable for active purified m6A demethylase enzyme and 90 min incubation is required for nuclear extract.

13. Remove the reaction solution from each well. Wash each well three times with 150 µl of the diluted 1X Wash Buffer each time.

8.2 Antibody Binding & Signal Enhancing:

1. Add 50 µl of the diluted Capture Antibody to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.
2. Remove the diluted Capture Antibody solution from each well.
3. Wash each well three times with 150 µl of the diluted 1X Wash Buffer each time.
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.
5. Remove the diluted Detection Antibody solution from each well.
6. Wash each well four times with 150 µl of the diluted 1X Wash Buffer each time.
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.
8. Remove the diluted Enhancer Solution from each well.
9. Wash each well five times with 150 µl of the diluted 1X Wash Buffer each time.

ΔNote: 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).

8.3 Signal Detection:

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 undemethylated m6A.
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 SS 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.

ΔNote: 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.

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

Table1. 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.

AS = Assay Standard

9. Data Analysis

Calculate the average for duplicate readings for sample wells and blank wells.

Calculate enzyme activity or inhibition using the following formulas:

Simple calculation:

$$Demethylase\ activity\ (OD/h/mg) = \frac{[OD\ (control\# - blank) - OD\ (sample - blank}{[Protein\ Amount(\mu g)/1000] * X\ Hour\ **}$$

#Control wells without nuclear extracts

*Protein amount added into the reaction at Step 8.1.9

**Incubation time at Step 8.1.12 (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)

$$Activity = \frac{(0.65 - 0.05)}{(5 \text{ x } 60)} \text{ x } 1000 = 2 \text{ OD/min/mg}$$

Accurate or specific activity calculation:

First generate standard curve and plot the OD values versus the amount of AS 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 DNA demethylase activity using the following formula:

$$Activity\ (ng/h/mg) = \frac{[OD\ (control\# - blank) - OD\ (sample - blank)]}{Slope \text{ x Protein Amount } (\mu g) * \ X\ Hour\ **} \text{ x } 1000$$

#Control wells without nuclear extracts

*Protein amount added into the reaction at Step 8.1.9

**Incubation time at Step 8.1.12 (in hours)

Inhibition calculation:

$$Inhibition\ \% = \left(1 - \frac{[OD\ (control - blank) - OD\ (inhibitor\ sample - blank)]}{[OD\ (control - blank) - OD\ (\ no\ inhibitor\ sample - blank)]}\right) \text{ x } 100\%$$

10. Typical Data

Data provided **for demonstration purposes only**.

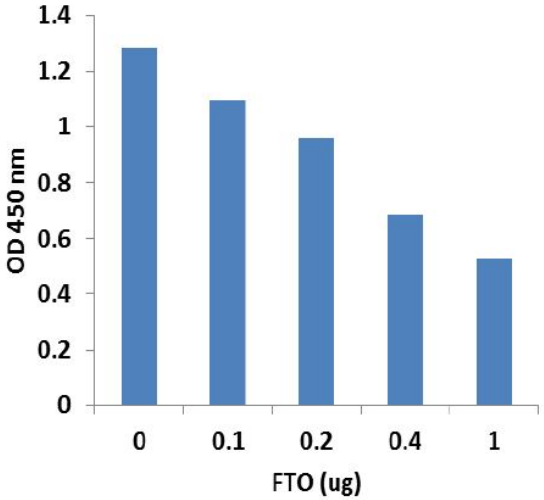


Figure 1. m^{6}A demethylase activity achieved by using recombinant FTO with the m6A Demethylase Assay Kit. Recombinant human FTO enzyme was added at different concentrations. Original OD data. OD is inversely proportional to FTO enzyme activity.

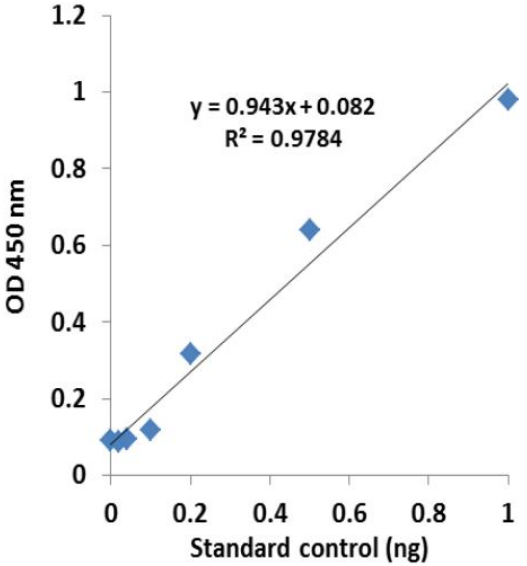


Figure 2. Illustrated standard curve generated using the m6A demethylase Assay Standard.

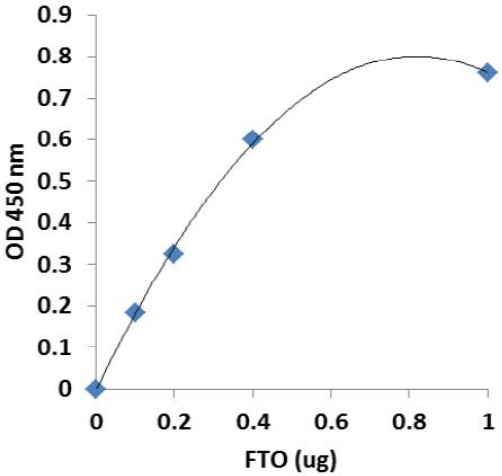


Figure 3. OD data after conversion from Figure 1.

11. FAQs / Troubleshooting

Problem	Reason	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 substrate and standard are not properly bound to the wells.	Ensure that (1) the m6A Substrate and Assay Standard are added into the wells; (2) the wells are completely covered with sufficient Binding Solution; and (3) binding time is sufficient (90 min).
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	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 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 8.1.3.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Materials supplied and Storage guidance in this User Guide for storage of Assay Standard.
High background present in the blank wells	Insufficient washing of wells.	Check if washing at each step are 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 8.2.4 should not exceed 45 minutes.
	Over development of color.	Decrease the development time in Step 8.3.1 before adding Stop Solution in Step 8.3.2
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. 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 8.1.10 and 8.1.11. 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 DNA demethylase 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.

12. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | 400 921 0189 / +86 21 2070 0500
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abc.com | +64-(0)9-909-7829