# Epigenase™ m6A Demethylase Activity/Inhibition Assay Kit (Colorimetric)

Base Catalog # P-9013

## PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

**Uses:** The Epigenase™ m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric) is suitable for measuring the activity/inhibition of total m$^6$A demethylases using nuclear extracts or purified m$^6$A demethylases like FTO and ALKBH5 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 m$^6$A DNA demethylases 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 m$^6$A demethylase activity. Because m$^6$A demethylase 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.

*110 Bi County Blvd. Ste. 122, Farmingdale, NY 11735*
*Tel: 1-877-374-4368 ■ Fax: 1-718-484-3956 ■ E-mail: info@epigentek.com ■ Web: www.epigentek.com*

## KIT CONTENTS

| Component | 48 Assays Cat. #P-9013-48 | 96 Assays Cat. #P-9013-96 | Storage Upon Receipt |
|---|---|---|---|
| **WB** (10X Wash Buffer) | 14 ml | 28 ml | 4°C |
| **DB** (Demethylase Buffer) | 3 ml | 6 ml | RT |
| **MS** (10 X $m^6A$ Substrate)* | 10 µl | 20 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 |
| **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 |

** 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 **MS**, **AS**, **DA**, **ES, Co-Factor 1**, **Co-Factor 2**, and **Co-Factor 3** 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 (**DB, 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
- ☐ 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 Epigenase™ m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric) is tested against predetermined specifications to ensure consistent product quality. EpigenTek 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:** EpigenTek 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. Be sure to use the latest User Guide for this kit which can be accessed online at www.epigentek.com/datasheet.

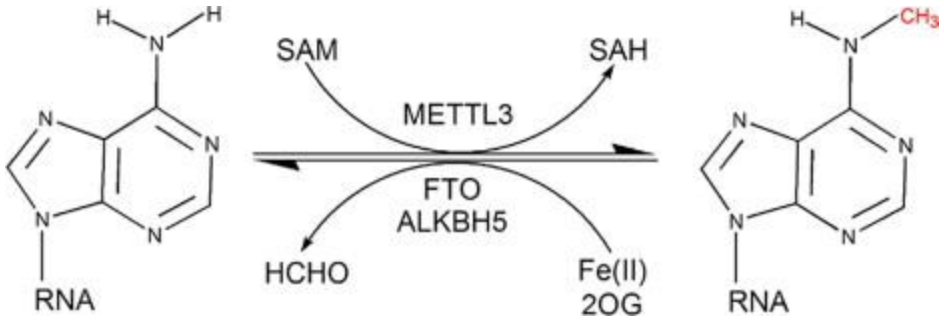
**Usage Limitation:** The Epigenase™ m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic application

**Intellectual Property:** The Epigenase™ m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric) and methods of use contain proprietary technologies by EpigenTek.

## A BRIEF OVERVIEW

N6-methyladenosine (m6A) is the most common and abundant modification on RNA molecules present in eukaryotes. Recently, DNA m6A is also identified in multicellular eukaryotes including *Caenorhabditis elegans* and *Drosophila melanogaster*, and furthermore identified in higher eukaryotes including plants, mouse and human cells**.** m$^6$A plays crucial roles in regulating DNA replication, DNA damage, RNA splicing, transposition, transcription, and cellular defense. In human cells, the m$^6$A modification is probably catalyzed by a methyltransferase complex METTL3/METTL14 and removed by the α-ketoglutarate (α-KG)- and $Fe^{2+}$-dependent dioxygenases such as FTO, ALKBH5 and TET-like enzymes. It was shown that METTL3 and α-KG /$Fe^{2+}$-dependent dioxygenases play important roles in many biological processes, ranging from development and metabolism to fertility. The dynamic and reversible chemical m$^6$A modification on DNA/RNA may also serve as a novel epigenetic marker of profound biological significance. Down-regulation of m$^6$A modification was first characterized in human cancer cells and tissues, relative to their normal controls. m$^6$A is found to be the most regulated DNA modification in cancers. In addition to the regulation in cancer cells, relative to

the primary cell/tissues which contain quite low DNA m$^6$A (<0.001%), a hundreds-fold increase of m$^6$A modification was found for *in vitro* cultured human cells (0.03%-0.22%).




▲ **Reversible m$^6$A methylation in DNA/RNA**

The m$^6$A decrease in cancer could be associated with m$^6$A demethylases. It was observed that FTO is highly expressed in acute myeloid leukemia (AM) and plays a critical oncogenic role in AML. Thus, determination of such enzymes would be important in benefiting cancer diagnositcs and developing new target-based cancer therapeutics. However, there are few methods available for detecting m$^6$A demethylase activity using both nuclear extracts and purified enzymes. To address this issue, EpigenTek developed and offers the m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric). 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 5 hours.
- Innovative kit composition enables background signals to be extremely low and allows the assay to be simple, accurate, reliable, and consistent.
- Both cell/tissue nuclear extracts and purified proteins can be used, which allows detection of inhibitory effects of m$^6$A demethylase inhibitor *in vivo* and *in vitro*.
- Novel assay principle allows high sensitivity to be achieved. The activity can be detected from as low as 2 µg of nuclear extracts or 50 ng of purified enzymes.
- The assay standard is included, which allows the specific activity of m$^6$A demethylase to be quantified.
- Strip-well microplate format makes the assay flexible for manual or high throughput analysis.

## PRINCIPLE & PROCEDURE

The Epigenase™ m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric) is designed for measuring total m$^6$A demethylase activity/inhibition. In an assay with this kit, the unique m$^6$A substrate is stably coated on the strip wells. Active m$^6$A demethylases bind to and demethylate m$^6$A contained in the substrate. The un-demethylated m$^6$A in the substrate can be recognized with a high affinity m$^6$A antibody and the immuno-signal is enhanced with enhancer solution. The ratio or amount of un-demethylated m$^6$A, which is inversely proportional to enzyme activity, can then be colorimetrically quantified through an ELISA-like reaction.

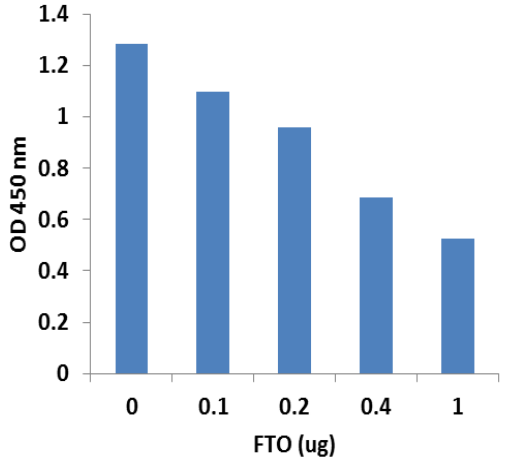



**Fig. 1.** m$^6$A demethylase activity achieved by using recombinant FTO with the Epigenase™ m$^6$A Demethylase Activity/Inhibition Assay Kit (Colorimetric). Recombinant human FTO enzyme was added at different concentrations. Original OD data. OD is inversely proportional to FTO enzyme activity.

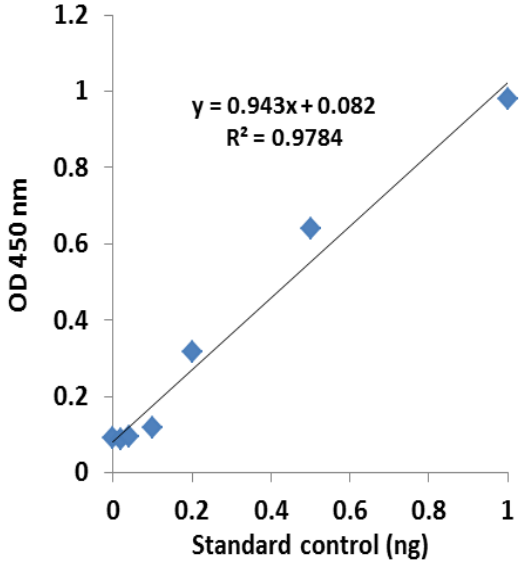



**Fig. 2.** Illustrated standard curve generated with the m$^6$A demethylase assay standard.

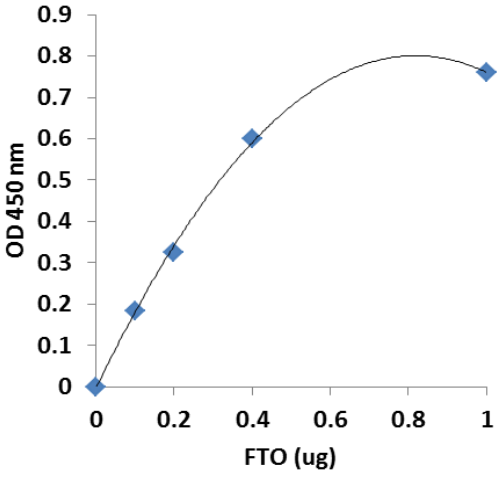



**Fig. 3.** OD data after conversion from Fig.1.

## 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. EpigenTek offers a nuclear extraction kit (Cat # OP-0002) 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 **Final DB**:

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

c. Prepare **1 X MS** Substrate:

Add 1 µl of **MS** (10X $m^6A$ Substrate) to 9 µl of **DB**. About 2 µl of **1 X MS** will be required for each assay well.

d. 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.

e. Prepare **Diluted DA**:

Dilute **DA (**Detection Antibody) with **Diluted WB** (1X Wash Buffer) at a ratio of 1:2000 (i.e., add 1 µl of **DA** to 2000 µl of **Diluted WB** (1X Wash Buffer). About 50 µl of **Diluted DA** will be required for each assay well.

f. Prepare **Diluted ES**:

Dilute **ES** (Enhancer Solution) with **Diluted WB** (1X Wash Buffer) at a ratio of 1:5000 (i.e., add 1 µl of **ES** to 5000 µl of **WB** (1X Wash Buffer). About 50 µl of **Diluted ES** will be required for each assay well.

g. Prepare **Diluted AS**:

Suggested Standard Curve Preparation: First, dilute **AS** with **DB** to 1 ng/µl by adding 5 µl of **AS** to 5 µl of **DB**. Then, further prepare five concentrations by combining the 1 ng/µl **Diluted AS** with **DB** 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 | AS (1 ng/µl) | DB | 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 **1X MS** into each sample well and control wells without nuclear extracts. Add 2 µl of **DB** into blank well. Add 1 µl of **Diluted AS** into the standard curve wells (see the designated wells depicted in Table 1 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 three times with 150 µl of the **Diluted WB** (1X Wash Buffer) each time.

g. Blank Wells: Add 50 µl of **Final DB** to each blank well.

h. Standard Wells: Add 50 µl of **Final DB** to each standard well.

i. Control Wells without Nuclear Extracts: Add 45-48 µl of **DB** and 2-5 µl of your protein extraction buffer.

j. Sample Wells Without Inhibitor: Add 46 to 49 µl of **Final DB** 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.

k. Sample Wells With Inhibitor: Add 41 to 44 µl of **Final DB**, 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 **DB** at a 1:10 ratio (i.e., add 0.5 µl of inhibitor to 4.5 µl of **DB**) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

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

***Note**: (1) The incubation time may depend on intrinsic enzyme activity. However, in general, 60 min incubation is suitable for active purified $m^6A$ demethylase enzyme and 90 min incubation is required for nuclear extract.*

m. 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. Remove the **Diluted CA** solution from each well.

c. Wash each well three times with 150 µl of the **Diluted WB (**1X Wash Buffer) each time.

d. Add 50 µl of the **Diluted DA** to each well, then cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

e. Remove the **Diluted DA** solution from each well.

f. Wash each well four times with 150 µl of the **Diluted WB (**1X Wash Buffer) each time.

g. Add 50 µl of the **Diluted ES** to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 min.

h. Remove the **Diluted ES** solution from each well.

i. Wash each well five 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** 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** solution will turn blue in the presence of sufficient undemethylated m6A.

b. Add 100 µl of **SS** 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.

***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. m$^6$A Demethylase 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:

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

# Control wells without nuclear extracts
* Protein amount added into the reaction at Step 2j.
** Incubation time at Step 2l (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)

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

For accurate or specific activity calculation:

First, generate a 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 m$^6$A DNA demethylase activity using the following formula:

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

# Control wells without nuclear extracts
* Protein amount added into the reaction at Step 2j
** Incubation time at Step 2l (in hours).

For inhibition calculation:

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

## 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 |
| **DB** | 50 µl | 400 µl | 800 µl | 2400 µl | 4800 µl |
| **1 X MS** | 2 µl | 16 µl | 32 µl | 96 µl | 192 µl |
| **AS** | N/A | N/A | 4 µl (optional) | 8 µl | 8 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** | Control | Control | Sample | Sample | Sample | Sample |

## TROUBLESHOOTING

| Problem | Possible Cause | Suggestion |
|---|---|---|
| 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 **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 3d 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 EpigenTek's Nuclear Extraction Kit (Cat. No. OP-0002). 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 2j and 2k. 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 $m^6A$ 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 (e.g., from well A to H or from well 1 to 12). |

## RELATED PRODUCTS

**RNA Methylation**

P-9005 EpiQuik™ m6A RNA Methylation Quantification Kit (Colorimetric)
P-9008 EpiQuik™ m6A RNA Methylation Quantification Kit (Fluorometric)
P-9010 MethylFlash™ m6A DNA Methylation ELISA Kit (Colorimetric)
P-9015 MethylFlash™ Urine N6-methyladenosine (m6A) Quantification Kit (Colorimetric)

**DNA Methyltransferase & Demethylase**

P-3009 EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric)
P-3086 Epigenase™ 5mC-Hydroxylase TET Activity/Inhibition Assay Kit (Colorimetric)
P-3094 Epigenase™ Thymine DNA Glycosylase (TDG) Activity/Inhibition Assay Kit (Colorimetric)