Introduction and Background

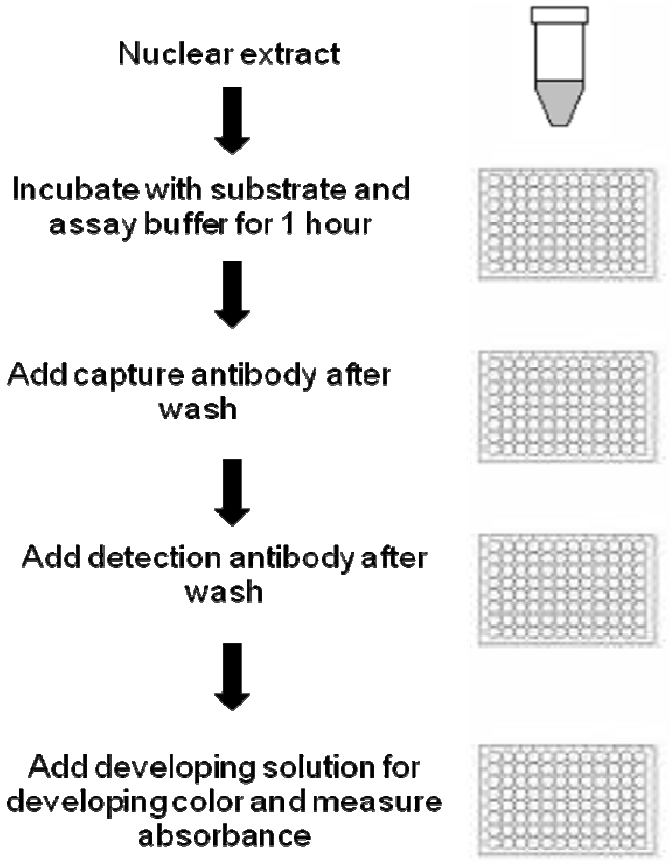
A. Overview

Epigenetic inactivation of genes plays a critical role in many important human diseases, especially in cancer. A major mechanism for epigenetic inactivation of the genes is methylation of CpG islands in genome DNA caused by DNA methyltransferases. Histone methyltransferases (HMTs) control or regulate DNA methylation through chromatin-dependent transcription repression or activation. HMTs transfer 1-3 methyl groups from S-adenosyl-L-methionine to the lysine and arginine residues of histone proteins. Inhibition of HMTs may lead to expression of the silenced genes and HMT inhibitors are currently developed for various therapeutic or experimental applications. G9a and polycomb group enzyme such as EZH2 are histone methyltransferases that catalyze methylation of histone H3 at lysine 27 (H3-K27) in mammalian cells. H3-K27 methylation mediates heterochromatin formation by forming a binding site for polycomb and also participates in silencing gene expression at euchromatic sites. Increased global H3- K27 methylation is also found to be involved in some pathological processes such as cancer progression. There is no method currently used for measuring HMT activity/inhibition (H3-K27). The Histone Methyltransferase Activity/ Inhibition Assay Kit (H3-K27) addresses this problem by using a unique procedure to measure HMT activity/inhibition (H3-K27). The kit has the following features:

- Quick and efficient, which can be finished within 3 hours.
- Innovative colorimetric assay with no need for radioactivity, electrophoresis, and chromatography.
- Specific measurement of activity/inhibition of H3-K27 histone methyltransferases.
- Strip microplate format makes the assay flexible: manual or high throughput analysis.
- Simple, reliable, and consistent assay conditions.

B. Test Principle

The Histone Methyltransferase Activity/Inhibition Assay Kit (H3-K27) is designed for measuring HMTs that specifically target histone H3 at lysine 27. In an assay with this kit, the histone substrate is stably captured on the strip wells through biotin-streptavidin binding. HMT enzymes transfer a methyl group to histone H3 substrate from Adomet to methylate the substrate at lysine 4. The methylated histone H3-K27 can be recognized with a high-affinity antibody. The ratio or amount of methylated H3-K27, which is directly proportional to enzyme activity, can be quantified through HRP conjugated secondary antibody-color development system. The HMT activity is then calculated based on the amount of methylated H3-K27 converted by the HMTs.



C. Notice for Application of Kit

- ✓ This kit has been configured for research use only and is not for diagnostic and clinical use.
- ✓ Suitable lab coat, disposable gloves and eye protection is required when working with the kit.

D. Application

For specifically measuring the activity/inhibition of individual histone methyltransferase targeting to lysine residues at different sites.

Material and Method

A. List of component

Component	Size
HE1 (10X wash buffer)	22 ml
HE2 (histone assay buffer)	3 ml
HE3 (Adomet)*	50 µl
HE4 (biotinylated substrate, 25 µg/ml)*	200 µl
HE5 (HMT standard, 10 µg/ml)*	20 µl
HE6 (capture antibody, 100 µg/ml)*	50 µl
HE7 (detection antibody, 100 µg/ml)*	20 µl
HE8 (developing solution)	12 ml
HE9 (stop solution)	6 ml
Control enzyme (150 µg/ml)*	20 µl
8 well assay strip (with frame)	12

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

B. Additional Required Materials But Not Provided

1. Orbital shaker
2. Pipettes and pipette tips
3. Microplate reader
4. 1.5 ml microcentrifuge tubes
5. Distilled water

C. Stability and storage

Upon receipt, store HE3, HE4, HE5, HE7, and control enzyme at –20℃ away from light. Store all other components at 4℃ away from light. The components of the kit should be stable for 6 months when stored properly.

D. Protocol

1. Prepare nuclear extracts by using your own successful method. Nuclear extracts can be used immediately or stored at –80℃ for future use.
2. Determine the number of strip wells required. Leave these strips in the plate frame (remaining unused strips can be put back in the bag. Seal the bag tightly and store at 4℃). Dilute 10 X HE1 with distilled water (pH 7.2-7.4) to the 1 X HE1.
3. Dilute HE3 with HE2 (at 1:5 ratio). Add 24 µl of HE2, 1.5 µl of the diluted HE3 and 2 µl of HE4 to each strip well. Then add 3 µl of nuclear extracts (4-20 µg) or HMT enzymes, mix and cover the strip wells with

Parafilm M and incubate at 37ºC for 60 min.

For HMT inhibition, add 3 µl of tested inhibitors at different concentrations and reduce HE2 volume to 21 µl. For blank, add 3 µl of HE2 instead of nuclear extracts. For standard curve, add 3 µl of HE2 instead of nuclear extracts and add 2 µl of HE5 at different concentrations (ex: 0.2 - 10 ng/µl) instead of HE4. A positive control can be optionally set up by adding 2-3 µl of control enzyme instead of nuclear extracts.

4. Aspirate and wash each well with 150 µl of 1 X HE1 three times.
5. Dilute the HE6 (at 1:100-1:200 ratio) with 1 X HE1. Add 50 µl of diluted HE6 to each strip well and incubate at room temperature for 60 min on an orbital shaker (50-100 rpm).
6. Aspirate and wash each well with 150 µl of 1 X HE1 four times.
7. Dilute the HE7 (at 1:1000 ratio) with 1 X HE1. Add 50 µl of diluted HE7 to each strip well and incubate at room temperature for 30 min
8. Aspirate and wash each well with 150 µl of 1 X HE1 four times. Allow 3 min for last wash.
9. Add 100 µl of HE8 into the wells and incubate at room temperature for 2-10 min away from light. Monitor color development in the sample and standard well (blue).
10. Add 50 µl of HE9 to each well to stop enzyme reaction when color in the standard wells containing the higher concentrations of standard control turns medium blue. The color should change to yellow and absorbance can be read on a microplate reader at 450 nm within 2-15 min.
11. Calculate HMT activity or inhibition. For simple calculation:

$$\text{Activity (O.D./h/mg)} = \frac{\text{OD (no inhibitor – blank)}}{\text{Protein amount } (\mu g)^{*} \times \text{hour}^{**}} \times 1000$$

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

For accurate calculation, plot OD value versus amount of HE5 and determine the slope as delta OD/ng.

Calculate HMT activity using the following formula:

$$\text{Activity (ng/h/mg)} = \frac{\text{OD (sample – blank)}}{\text{Protein amount } (\mu g) \times \text{hour} \times \text{slope}} \times 1000$$

* Protein amount added into the reaction at step 3.

** Incubation time at step 3.

Troubleshooting

- **No Signal for Both the Positive Control and the Samples**

Reagents are added incorrectly.	Check if reagents are added in order and if some steps of the procedure are omitted by mistake.
Incubation time and temperature is incorrect.	Ensure the incubation time and temperature in the protocol is correctly followed.

- **No Signal or Very Weak Signal for Only the Positive Control**

The positive control enzyme is insufficiently added to the well.	Ensure sufficient amount of control enzyme is added.
The positive control enzyme has lost activity due to incorrect storage.	Follow the guidance in the protocol for storage of positive control.

- **No Signal for Only the Sample**

The protein sample is not properly extracted.	Ensure the nuclear protein extraction protocol is suitable for HMT protein extraction. Sodium chloride concentration of the extraction buffer should not be more than 100 mM.
The protein amount is added into well insufficiently.	Ensure extract contains enough amount of proteins.
The sample is not prepared from fresh cells or tissues.	The nuclear extracts from frozen cells or tissues significantly loss enzyme activity. The fresh sample should be used.
Nuclear extracts are incorrectly stored or have been stored for a long duration.	Ensure the nuclear extracts are stored at –80℃ for no more than 6 weeks.
Absence of HMT activity in the sample due to treatment.	N/A

- **High Background Present for the Blank**

The well is not washed enough.	Check if wash at each step is performed according to the protocol.
Contaminated by the positive control or HMT standard.	Ensure the well is not contaminated by adding the control enzyme or HMT standard accidentally, or by using enzyme or HMT standard contaminated tips .
Overdevelopment.	Decrease development time in protocol step 9 of “target protein level detection.”