Introduction and Background

A. Overview

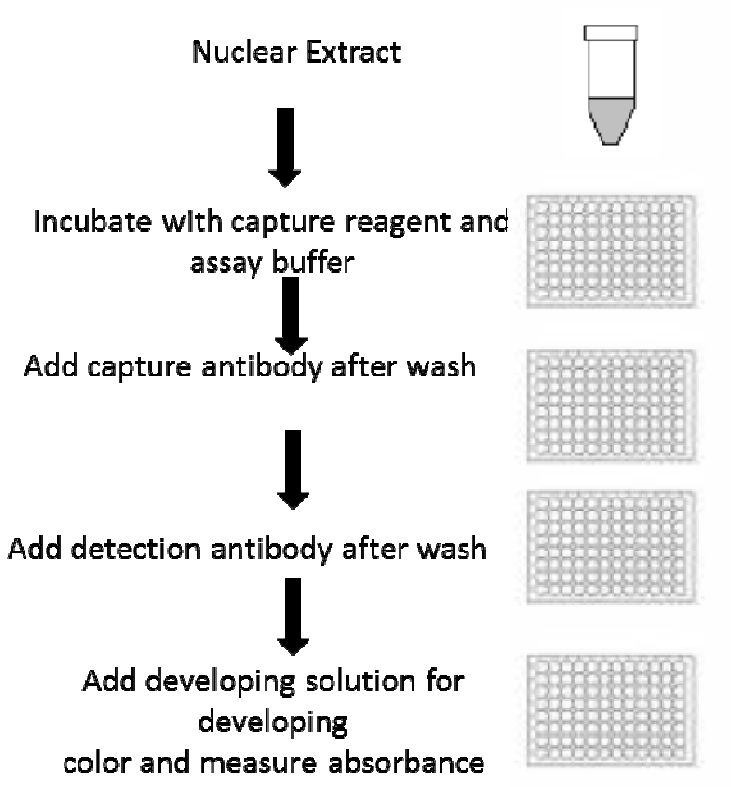
Epigenetic inactivation of genes plays a critical role in many important human diseases, especially in cancer. A core mechanism for epigenetic inactivation of the genes is methylation of CpG islands in genome DNA. Methylation of CpG islands involves the course in which DNA methyltransferases (Dnmts) transfer a methyl group from S-adenosyl-L-methionine to the fifth carbon position of the cytosines. At least three families of Dnmts have been identified in mammals now: Dnmt1, Dnmt2, and Dnmt3. The Dnmt3 family comprises of three different proteins: Dnmt3A, Dnmt3B, and Dnmt3L. Dnmt3A and Dnmt3B have been demonstrated to methylate both unmethylated and hemimethylated DNA equally and supposed to mediate de novo methylation together with Dnmt1. Increased activation or amount of Dnmt3 is believed to be involved in carcinogenesis, and other genetic and epigenetic diseases.

Several methods such as Western blot are used for measuring levels of Dnmt3A. However these methods available so far are inconvenient, considerably time consuming, labor intensive, or have low throughput. The Dnmt3A Assay Kit addresses these problems by using a unique procedure to measure the amount of Dnmt3A. The kit has the following features:

- Extremely fast procedure, which can be completed within 3 hours.
- Innovative colorimetric assay, which quantitatively measures Dnmt3A amount with no need for electrophoresis.
- Strip microplate format makes the assay flexible: manual or high throughput analysis.
- Simple, reliable, and consistent assay conditions.

B. Test Principle

The Dnmt3A Assay Kit is designed for measuring total Dnmt3A amount from tissues or cells. In an assay with this kit, the unique Dnmt affinity substrate is stably coated on the strip well. The sample is added into the well and Dnmt3A contained in the sample binds to the substrate. The bound Dnmt3A can be recognized with specific Dnmt3A antibody and colorimetrically quantified through an ELISA-like reaction. The amount of Dnmt3A is proportional to the intensity of color development.



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 measuring Dnmt amount quantitatively from fresh tissue and cultured cells of human and mouse.

Material and Method

A. List of component

	96 assays
DA1 (10X wash buffer)	**25 ml**
DA2 (assay buffer)	**3 ml**
DA3 (Dnmt3A standard, 20 µg/ml)*	**20 µl**
DA4 (affinity antibody 100 µg/ml)*	**50 µl**
DA5 (detection antibody 200 µg/ml)*	**20 µl**
DA6 (developing solution)	**12 ml**
DA7 (stop solution)	**6 ml**
8 well substrate-coated 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

C. Stability and storage

Store DA3 and DA5 at –20℃ away from light. Store DA4, DA6 and 8 well assay strips at 4℃ away from light. Store other components at room temperature. All components are stable for 6 months from date of shipment in proper storage.

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 placed back in the bag.
3. Seal the bag tightly and store at 4℃). Dilute 10 X DA1 with distilled water (pH 7.2-7.5) to 1 X DA1.
4. Add 27 µl of DA2 and then add 3 µl of nuclear extracts (4-10 µg) or purified Dnmt3A protein to each strip well. Mix and cover the strip wells with Parafilm M and incubate at 37℃ for 60 min. For blank, add 3 µl of DA2 instead of nuclear extracts. For standard curve, add 3 µl of DA3 at different amount (1-20 ng) instead of nuclear extract.
5. Aspirate and wash each well with 150 µl of 1 X DA1 three times.
6. Dilute the DA4 (at 1:100 ratio) to 1 µg/ml with 1 X DA1. Add 50 µl of diluted DA4 to each strip well and

incubate at room temperature for 60 min on an orbital shaker (50-100 rpm).

7. Aspirate and wash each well with 150 µl of 1 X DA1 four times.
8. Dilute the DA5 (at 1:1000 ratio) with 1 X DA1. Add 50 µl of diluted DA5 to each strip well and incubate at room temperature for 30 min.
9. Aspirate and wash each well with 150 µl of 1 X DA1 five times. Allow 3 min for last wash.
10. Add 100 µl of DA6 to each well and incubate at room temperature for 2-10 min away from light. Monitor color development in the sample and control well (blue).
11. Add 50 µl of DA7 to each well and read absorbance on microplate reader at 450 nm.
12. Calculate Dnmt3A amount:

Plot OD value versus amount of DA3 and determine the slope as delta OD/ng.

Calculate Dnmt3A amount using the following formula:

$$\text{Dnmt3B (ng/ml)} = \frac{\text{OD (sample – blank)}}{\text{slope}} \times \text{sample dilution}^*$$

* If no dilution before adding protein extracts (3 µl) into the well, the sample dilution factor should be 333 (1000:3).

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 are correctly followed.

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

The standard protein is insufficiently added to the well.	Ensure sufficient amount of enzyme is added.
The Dnmt1 standard has lost activity due to incorrect storage.	Follow the guidance in the protocol for storage of Dnmt3A standard.

- **No Signal for Only the Sample**

The protein amount is added into well insufficiently.	Ensure extract contains sufficient amount of proteins.
Nuclear extracts are incorrectly Nuclear extracts are incorrectly a long period.	Ensure the nuclear extracts are stored at –80℃.

- **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 Dnmt3A standard.	Ensure the well is not contaminated by adding Dnmt3A standard accidentally or by using Dnmt3A contaminated tips.
Overdevelopment.	Decrease development time in protocol step 9.