ab113472 – DNA Demethylase (total) Activity Quantification Kit (Colorimetric)

Instructions for Use

For the measurement of DNA demethylase activity/inhibition using purified enzyme or protein extracts from cultured cells and tissues

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

Version 2 Last Updated 5 February 2019

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1. BACKGROUND

DNA methylation is known to play an essential role in all biological processes through the repression of transcription and development. Hypermethylation of CpGs in the promoters of tumor suppression gene has been demonstrated to cause the epigenetic silence of these genes and constitutes a common feature of many cancers. In contrast, DNA demethylation is necessary for the epigenetic reprogramming of the genes and involves the processes of many important diseases such as tumor progression. Demethylation of DNA can either be passive or active, or a combination of both. Active demethylation of DNA requires specific demethylase participation such as MBD2.

There is only the radioisotopic method currently available for measuring DNA demethylase activity/inhibition, which is time consuming, labor-intensive, and has low throughput and produces radioactive waste. The DNA Demethylase Activity/Inhibition Assay Kit addresses these problems by using a unique procedure to measure DNA demethylase. The principle of the assay is based on increased DNA demethylase activity causing the reduction of methylated DNA, which can be detected immunologically, and is proportional to the colorimetric intensity.

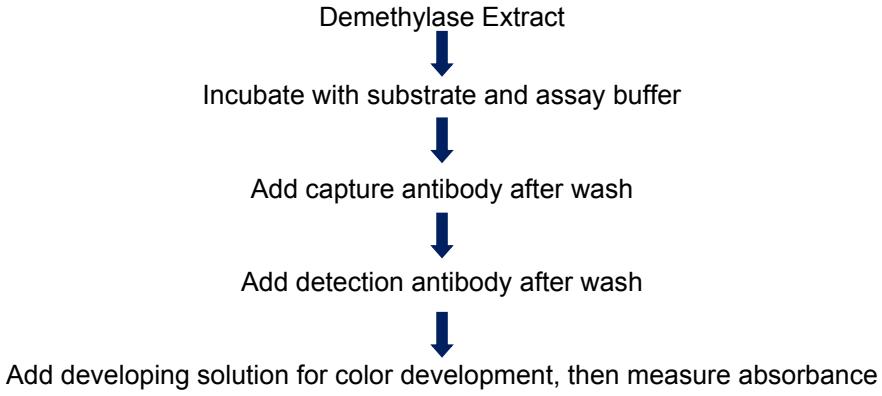
The kit has the following features:

- Quick and efficient procedure, which can be finished within 4 hours
- Innovative colorimetric assay without the need for radioactivity, electrophoresis, or chromatography
- Strip microplate format makes the assay flexible: manual or high throughput analysis
- Simple, reliable, and consistent assay conditions

The DNA Demethylase Activity/Inhibition Assay Kit is designed for measuring total DNA demethylase activity/inhibition. In an assay with this kit, the unique methylated DNA substrate is stably captured on the strip

wells. Active DNA demethylases bind to and demethylate DNA substrate. The methylated DNA can be recognized with a high affinity 5-methylcytosine antibody. The ratio or amount of methylated DNA, which is inversely proportional to enzyme activity, can then be colorimetrically quantified through an ELISA-like reaction.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit as given in the table upon receipt and away from light.

Observe the storage conditions for individual prepared components in sections 9 & 10.

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

Check if 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.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	11 mL	22 mL	4°C
Demethylase Assay Buffer	3 mL	6 mL	RT
Demethylation Substrate*	0.1 mL	0.2 mL	-20°C
Demethylation Standard (10 µg/mL)*	20 µL	40 µL	-20°C
Capture Antibody, 1000 µg/mL*	5 µL	8 µL	4°C
Detection Antibody, 200 µg/mL*	10 µL	20 µL	-20°C
Developing Solution	5 mL	10 mL	4°C
Stop Solution	3 mL	6 mL	RT
8-well assay strips (with 1 frame)	6	12	4°C
Substrate Binding Solution	5 mL	10 mL	RT

*Spin the solution down to the bottom prior to use.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Orbital shaker
- Pipettes and pipette tips
- Microplate reader
- 1.5 mL microcentrifuge tubes

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

8. TECHNICAL HINTS

- Avoid foaming or bubbles when mixing or reconstituting components
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions
- Ensure plates are properly sealed or covered during incubation steps
- Complete removal of all solutions and buffers during wash steps
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Prepare fresh reagents immediately prior to use.

9.1 **1X Wash Buffer**

Dilute 10X Wash Buffer with distilled water (pH 7.2-7.5) at a 1:10 ratio (e.g. 1 mL of 10X Wash Buffer + 9 mL distilled water).

9.2 **Diluted Demethylation Substrate**

Dilute Demethylation Substrate at a 1:80 ratio with Substrate Binding Solution.

9.3 **Diluted Capture Antibody Solution**

Dilute Capture Antibody (at a 1:1000 ratio) to 1 µg/mL with 1X Wash Buffer.

9.4 **Diluted Detection Antibody Solution**

Dilute Detection Antibody (at a 1:2000 ratio) with 1X Wash Buffer.

10. SAMPLE PREPARATION

Prepare protein extracts by using your own successful method. Abcam offers a series of Protein Extraction Kits which are optimized for extracting protein from Cultured cells and Tissues.

11. ASSAY PROCEDURE

11.1 Determine the number of strip wells required. Leave these strips in the plate frame (remaining unused strips can be placed back in the bag. Seal the bag tightly and store at 4°C).

11.2 Add 80 µL of the diluted Demethylation Substrate into each well, except the wells for the blank and standard curve. For Blank, add 80 µL of the Substrate Binding Solution. For preparation of the standard curve, add 80 µL of Substrate Binding Solution into the wells (without diluted Demethylation Substrate added), followed by adding 1 µL of Demethylation Standard at different amounts (0.05 – 5 ng). Cover the wells with Parafilm M and incubate at room temperature for 75 minutes.

11.3 Aspirate and wash each well with 150 µL of 1X Wash Buffer two times.

11.4 **For blank and standard wells:** Add 50 µL of Demethylase Assay Buffer.

11.5 **For the sample wells:** Add 45-48 µL of Demethylase Assay Buffer to each well, followed by adding 2-5 µL of the protein extracts (5-10 µg) or purified demethylase.

11.6 **For the no enzyme control wells:** Add 45-48 µL of Demethylase Assay Buffer and 2-5 µL of your protein extraction buffer or enzyme buffer.

11.7 **For inhibitor wells:** Add 42-45 µL of Demethylase Assay Buffer, 2-5 µL of protein extracts or enzyme and 3 µL of tested compounds at desired concentration. Mix and cover the strip wells with Parafilm M and incubate at 37°C for 1 hour.

11.8 Aspirate and wash each well with 150 µL of 1X Wash Buffer two times.

11.9 Add 50 µL of the diluted Capture Antibody to each well and incubate at room temperature for 60 minutes on an orbital shaker (50-100 rpm).

11.10 Aspirate and wash each well with 150 µL of 1X Wash Buffer three times.

11.11 Add 50 µl of the diluted Detection Antibody to each well and incubate at room temperature for 30 minutes.

11.12 Aspirate and wash each well with 150 µL of 1X Wash Buffer five times.

11.13 Add 100 µL of Developing Solution to each well and incubate at room temperature for 2-10 minutes away from light. Monitor the color development in the sample and control wells (blue).

11.14 Add 50 µL of Stop Solution to each well to stop enzyme reaction when the color in the control well turns medium blue. The color should change to yellow and absorbance can be read on a microplate reader at 450 nm within 2-15 minutes.

12. ANALYSIS

Calculate Demethylase activity and inhibition. For simple calculation, use the following formulae:

$$\text{Demethylase activity (OD/h/mg} = \frac{[\text{OD (control} - \text{blank)} - \text{OD (sample} - \text{blank)}]}{[\text{Protein Amount (ug)}/1000] * \times \text{Hour} **}$$

$$\text{Inhibition \%} = (1 - \frac{[\text{OD (control - blank) - OD (inhibitor sample - blank)}]}{[\text{OD (control - blank) - OD (no inhibitor sample - blank)}]}) \times 10$$

Calculate DNA demethylase activity using the following formula:

$$\text{Activity (ng/h/mg)} = \frac{[\text{OD (control} - \text{blank)} - \text{OD (sample} - \text{blank)}]}{\text{Slope} \times \text{Protein Amount (ug)} * \times \text{Hour} **} \times 1000$$

* Protein amount added into the reaction at section 11, step 11.5 in µg.

** Incubation time at section 11, step 11.9.

13. TROUBLESHOOTING

Problem	Cause	Solution
No Signal for the Sample	The protein sample is not properly extracted.	Ensure the nuclear protein extraction protocol is suitable for DNA methylase protein extraction.
	The protein amount is added into well insufficiently.	Ensure extract contains a sufficient amount of protein.
	The sample is prepared from frozen cells or tissues.	The nuclear extracts from frozen cells/tissue significantly lose enzyme activity. A fresh sample should be used.
	Nuclear extracts are incorrectly stored or have been stored for a long time.	Ensure the nuclear extracts are stored at –80°C for no more than 6 weeks.
	Absence of DNA methylase activity in the sample due to treatment.	N/A
High Background Present for the Blank	The well is not washed sufficiently.	Check if wash at each step is performed according to the protocol.
	Overdevelopment	Decrease development time.

14. NOTES

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