ab113468 – DNMT Activity Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of activity/inhibition of DNMT using nuclear extracts or purified enzymes from a broad range of species

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

Version 1 Last Updated 31 January 2019

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1. BACKGROUND

DNA methylation occurs by a covalent addition of a methyl group at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine. These methyl groups project into the major grooves of DNA and inhibit transcription. In human DNA, 5-methylcytosine is found in approximately 1.5% of genomic DNA, primarily at CpG sites. There are clusters of CpG sites at 0.3 to 2 kb stretches of DNA known as CpG islands that are typically found in or near promoter regions of genes, where transcription is initiated. In the bulk of genomic DNA, most CpG sites are heavily methylated. However, CpG islands in germ-line tissue and promoters of normal somatic cells remain unmethylated, allowing gene expression to occur. When a CpG island in the promoter region of a gene is methylated, the expression of the gene is repressed. The repression can be caused by directly inhibiting the binding of specific transcription factors, and indirectly by recruiting methyl-CpG-binding proteins and their associated repressive chromatin remodeling activity. In addition to the effect on gene transcription, DNA methylation is also involved in genomic imprinting, which refers to a parental origin specific expression of a gene, and the formation of a chromatin domain.

DNA methylation is controlled at several different levels in normal and diseased cells. The addition of methyl groups is carried out by a family of enzymes, DNA methyltransferases (DNMTs). Chromatin structure in the vicinity of gene promoters also affects DNA methylation and transcriptional activity. Three DNMTs (DNMT1, DNMT3A, and DNMT3B) are required for the establishment and maintenance of DNA methylation patterns. Two additional enzymes (DNMT2 and DNMT3L) may also have more specialized but related functions. DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation, while DNMT3A and DNMT3B seem to mediate the establishment of new or de novo DNA methylation patterns. DNMT3L is found to be a catalytically inactive regulatory factor of DNA methyltransferases, which is essential for the function of DNMT3A and DNMT3B. Diseased cells such as cancer cells may be different in that DNMT1 alone is not responsible for maintaining abnormal

gene hypermethylation and both, DNMT1 and DNMT3B, may be cooperative for this function. The local chromatin structure also contributes to the control of DNA methylation.

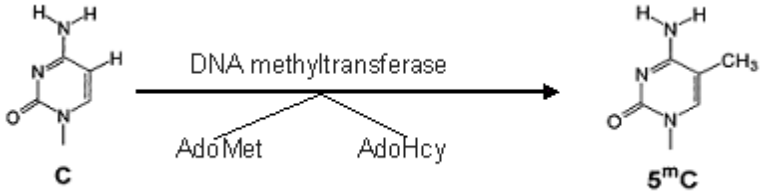


Figure 1. Methylation of cytosine in DNA via DNA methyltransferase and Sadenosylmethionine

The importance of DNA methylation is emphasized by the growing number of human diseases that are known to occur when DNA methylation information is not properly established and/or maintained. Abnormal DNA methylation associated with increased expression or the activity of DNMTs has been found in many different diseases, especially in cancer. Inhibition of DNMTs may lead to demethylation and expression of silenced genes. DNMT inhibitors are currently being developed as potential anticancer agents.

Conventional DNMT activity/inhibition assay methods are time consuming, labor-intensive, have low throughput, and/or produce radioactive waste. ab113468 addresses these issues using a simple method with an ELISA-like 96-well plate format and by enhancing sample signals and significantly minimizing background signals.

- Fluorometric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 3 hours and 45 minutes.
- Safe and innovative fluorometric assay without radioactivity, extraction, and chromatography.

- The ultra-sensitive detection limit can be as low as 0.2 µg of nuclear extract or 0.2 ng of purified enzymes, which is ten times better than the predecessor kit.
- Optimized antibody & enhancer solutions allow high specificity to 5-mC without cross-reactivity to unmethylated cytosine.
- 96 stripwell microplate format allows for either low or high throughput analysis.

The DNMT Activity Quantification Kit (Fluorometric) contains all reagents necessary for the measurement of DNMT activity or inhibition. In this assay, a universal DNMT substrate is stably coated onto microplate wells. DNMT enzymes transfer methyl group to cytosine from Adomet to methylate DNA substrate and the methylated DNA can be recognized with an anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be measured by reading the fluorescence in a fluorescent microplate spectrophotometer at 530 excitation and 590 emission. The activity of DNMT enzymes is proportional to the fluorescence intensity measured.

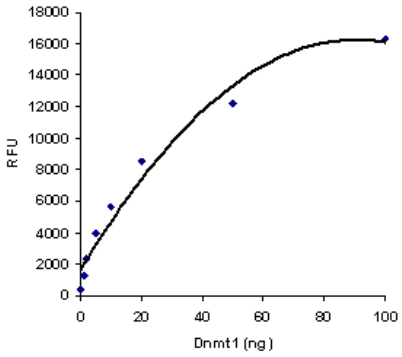
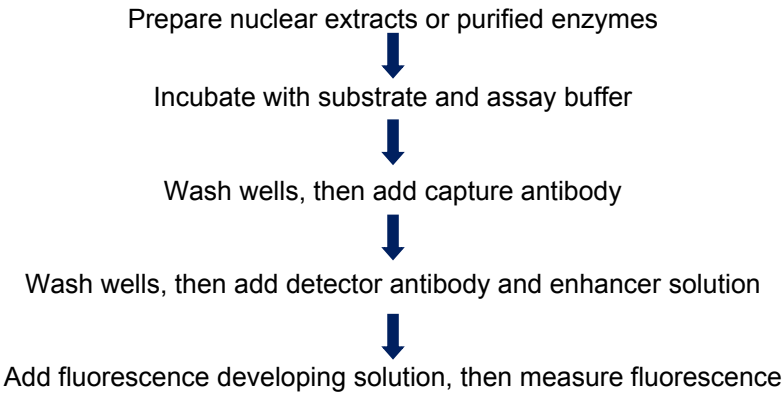


Figure 2. Demonstration of high sensitivity and specificity of DNMT activity/inhibition assay achieved by using recombinant DNMT1 with ab113468.

ab113468 is suitable for measuring total DNMT activity or inhibition using nuclear extracts or purified enzymes from a broad range of species such as mammalians, plants, fungi, bacteria, and viruses in a variety of forms including, but not limited to, cultured cells and fresh and frozen tissues. Nuclear extracts can be prepared by using your own successful method. For your convenience and the best results, Abcam also offers a nuclear extraction kit (ab113474) optimized for use with this kit. Nuclear extracts can be used immediately or stored at –80°C for future use. Purified enzymes can be active DNMTs from recombinant proteins or isolated from cell/tissues.

A positive enzyme control is provided in this kit. Because DNMT 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.

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	14 mL	28 mL	4°C
DNMT Assay Buffer	4 mL	8 mL	RT
Adomet, 50X*	60 µL	120 µL	-20°C
DNMT Enzyme Control, 50 µg/mL*	6 µL	12 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*	6 µL	12 µL	-20°C
Fluoro Developer*	6 µL	12 µL	-20°C
Fluoro Enhancer*	6 µL	12 µL	4°C
Fluoro Diluter	4 mL	8 mL	RT
Adhesive covering film	1	1	RT
8-well assay strips (with 1 frame)	6	12	4°C

*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:

- Adjustable pipette or multiple-channel pipette
- Multiple-channel pipette reservoirs
- Aerosol resistant pipette tips
- Fluorescence microplate reader capable of reading fluorescence at 530 excitation and 590 emission nm
- 1.5 ml microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract or purified enzyme samples containing DNMT activity
- Parafilm M or aluminium foil

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**

Add 13 mL of 10X Wash Buffer to 117 mL of distilled water and adjust pH to 7.2-7.5. This 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 **Adomet Working Buffer**

Freshly prepare the Adomet Working Buffer required for the assay by adding 2 µL of 50X Adomet into 98 µL of Assay Buffer. About 50 µL of this Adomet Working Buffer will be required for each assay well.

9.3 **Diluted Capture Antibody Solution**

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.

9.4 **Diluted Detection Antibody Solution**

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 this Diluted Detection Antibody will be required for each assay well.

9.5 **Diluted 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 this Diluted Enhancer Solution will be required for each assay well.

9.6 **Fluorescence Development Solution**

Add 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer to every 500 µL of Fluoro Diluter.

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.*

Note: *The DNMT Enzyme Control is an enzyme with activity of both maintenance and de novo DNMTs and is used as the positive control of the assay. We do not recommend using this enzyme control to generate a standard curve for quantifying the activity of your samples, as the amount of the enzyme is limited and catalytic activity/unit is different.*

Table 1. Approximate amount of required buffers and solutions for defined assay wells, based on the protocol.

Reagents	1 well	8 wells (1 strip)	16 wells (2 strips)	48 wells (6 strips)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL
Adomet Working Buffer	50 µL	400 µL	800 µL	2400 µL
Diluted Capture Antibody	50 µL	400 µL	800 µL	2400 µL
Diluted Detection Antibody	50 µL	400 µL	800 µL	2400 µL
Diluted Enhancer Solution	50 µL	400 µL	800 µL	2400 µL
Fluorescence Development Solution	0.05 mL	0.4 mL	0.8 mL	2.4 mL
Stop Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL
DNMT Enzyme Control	N/A	0.25 µL – 1 µL	0.5 µL – 2 µL	1 µL – 4 µL

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be between 0.5 µg and 20 µg with an optimal range of 5 µg – 10 µg. The amount of purified enzymes can be 0.5 ng – 200 ng, depending on the purity and catalytic activity of the enzymes.

Nuclear Extraction: You can use your own method of choice for preparing nuclear extracts. Nuclear extract or purified DNMT enzymes should be stored at –80°C until use.

11. PLATE PREPARATION

The suggested strip-well plate setup for the DNMT activity assay in a 48-assay format. The controls and samples can be measured in duplicates.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	Enzyme Control 0.5 µL	Enzyme Control 0.5 µL	Sample	Sample	Sample	Sample
C	Enzyme Control 1 µL	Enzyme Control 1 µL	Sample	Sample	Sample	Sample
D	Sample	Sample	Sample	Sample	Sample	Sample
E	Sample	Sample	Sample	Sample	Sample	Sample
F	Sample	Sample	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

12. ASSAY PROCEDURE

12.1 Enzymatic Reaction

12.1.1 Predetermine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and positive control) to ensure that the signal generated is validated. 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).

12.1.2 Blank Wells: Add 50 µL of Adomet Working Buffer per well.

12.1.3 Positive Control Wells: Add 50 µL of Adomet Working Buffer and 1 µL of DNMT Enzyme Control per well.

12.1.4 Sample Wells Without Inhibitor: Add 45 µL – 49 µL of Adomet Working Buffer, and 1 µL – 5 µL of nuclear extracts or 1 µL – 5 µL of purified DNMT enzymes per well. Total volume should be 50 µL/well.

12.1.5 Sample Wells With Inhibitor: Add 40 µL – 44 µL of Adomet Working Buffer, 1 µL – 5 µL of nuclear extracts or 1 µL – 5 µL of purified DNMT enzymes, and 5 µL of inhibitor solution per well. Total volume should be 50 µL/well.

Note: *Follow the suggested well setup diagrams in Section 11. It is recommended to use 5 µg – 10 µg of nuclear extract per well or 10 ng – 100 ng of purified enzyme per well.*

Note: *The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 µM – 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with Assay Buffer at a 1:10 ratio (e.g., add 0.5 µL of inhibitor to 4.5 µL of Assay Buffer), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.*

12.1.6 Tightly cover the strip-well microplate with the Adhesive Covering Film to avoid evaporation, and incubate at 37°C for 90-120 min.

Note: *The incubation time may depend on intrinsic DNMT activity. In general, 90 min incubation is suitable for active purified DNMT enzymes and 120 min incubation is required for nuclear extracts. The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used.*

12.1.7 Remove the reaction solution from each well. Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

12.2 **Antibody Binding & Signal Enhancing**

12.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.

12.2.2 Remove the Diluted Capture Antibody solution from each well.

12.2.3 Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

12.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

12.2.5 Remove the Diluted Detection Antibody solution from each well.

12.2.6 Wash each well with 150 µL of the 1X Wash Buffer each time for four times.

12.2.7 Add 50 µL of the Diluted Enhancer Solution to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.

12.2.8 Remove the Diluted Enhancer Solution from each well.

12.2.9 Wash each well with 150 µL of the 1X Wash Buffer each time for five times.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed as much as possible at each wash step.*

12.3 **Signal Detection**

12.3.1 Add 50 µl of Fluorescence Development Solution to each well and incubate at room temperature for 1 - 3 min away from direct light. The Fluorescence Solution will turn pink in the presence of sufficient methylated DNA.

12.3.2 Read the fluorescence on a fluorescence microplate reader within 2 - 10 min at Ex/Em = 530/590 nm.

Note: *If the stripwell microplate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

13. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells.

Calculate DNMT activity or inhibition using the following formula:

$$\text{DMNT activity (RFU/h/µg)} = \frac{\text{Sample RFU} - \text{Blank RFU}}{\text{(Protein Amount (µg)* x hour**)}} \times 1000$$

* Protein amount added into the reaction at section 12, step 12.1.4 in µg. ** Incubation time at section 12, step 12.1.6.

Example calculation:

Average RFU of sample is 5500

Average RFU of blank is 500

Protein amount is 5 µg

Incubation time is 2 hours (120 minutes)

DNMT activity = [(5500 – 500)/ (5 x 2)] x 1000
= 500,000 OD/h/µg

Calculate DNMT inhibition using the following formula:

$$\text{Inhibition (\%)} = 1 - \left(\frac{\text{Inhibitor sample RFU} - \text{Blank RFU}}{\text{No inhibitor sample RFU} - \text{Blank RFU}} \right) \text{X } 100\%$$

14. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the positive control 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 well is incorrectly washed before enzyme reaction.	Ensure the well is not washed prior to adding the positive control and sample.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol is followed correctly.
	Incorrect fluorescence reading.	Check if appropriate fluorescent wavelength (Ex/Em = 530/590 nm filter) is used.
No signal or weak signal in both the positive control and sample wells.	Kit was not stored or handled properly.	Ensure all components of the kit were stored at the appropriate temperature and caps are tightly capped after each opening or use.

Problem	Cause	Solution
No signal or weak signal in both the positive control and sample wells.	The DNMT enzyme control is insufficiently added to the well in Step 11.1.3.	Ensure a sufficient amount of DNMT enzyme control is added.
	The quality of the DNMT enzyme control has been degraded due to improper storage conditions.	Follow the Storage guidance in this User Guide for storage instructions of DNMT Enzyme Control.
High Background Present for the Blank	Insufficient washing of wells.	Check if washing recommendations at each step is performed according to the protocol.
	Contaminated by sample or positive control.	Ensure the well is not contaminated from adding sample or positive control accidentally or from using contaminated tips.
	Incubation time with detection antibody is too long.	The incubation time at step 12.2.4 should not exceed 45 min.
	Over development of fluorescence.	Decrease the development time in Step 12.3.1 and measure fluorescence as quickly as possible.

Problem	Cause	Solution
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for DNMT protein 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 purified enzymes or nuclear extracts is used as indicated in section 10. 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 –80ºC, with no more than 6 weeks for nuclear extracts and 6 months for purified enzymes. Avoid repeated freezing/thawing.

Problem	Cause	Solution
	Little or no activity of DNMT 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 or purified enzymes.
Uneven fluorescent development	Insufficient washing of the wells.	Ensure the wells are washed according to the protocol. Ensure any residues from the wash buffer are removed as much as possible.
	Delayed fluorescence development in the wells.	Ensure fluorescence development solution is added sequentially and consistent with the order you added the other reagents (e.g., from well A to well G or from well 1 to well 12).

15. NOTES

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