DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric)

Catalog Number KA1515

48 assays

Version: 5.1

Intended for research use only

www.abnova.com

Table of Contents

Introduction

Intended Use

The DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric) 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/frozen tissues. Nuclear extracts can be prepared by using your own successful method. For your convenience and the best results, we also offer a Nuclear Extraction Kit (Cat # KA6124) 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.

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.

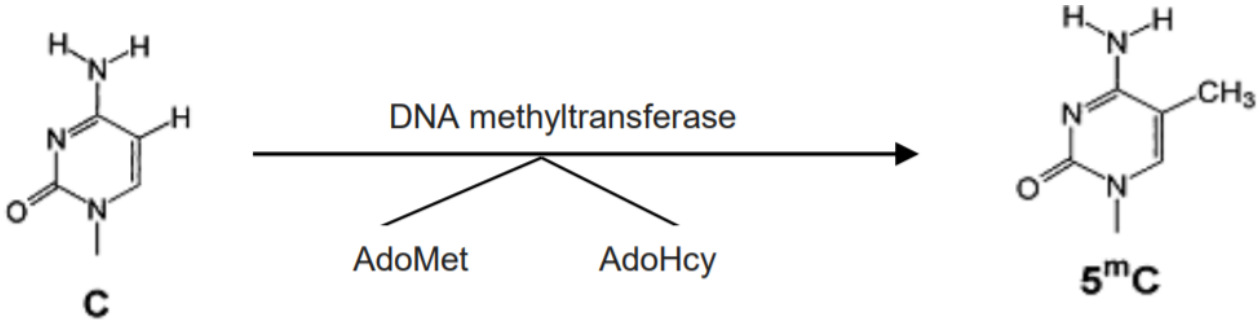


Fig 1. Methylation of cytosine in DNA via DNA methyltransferase and S-adenosylmethionine

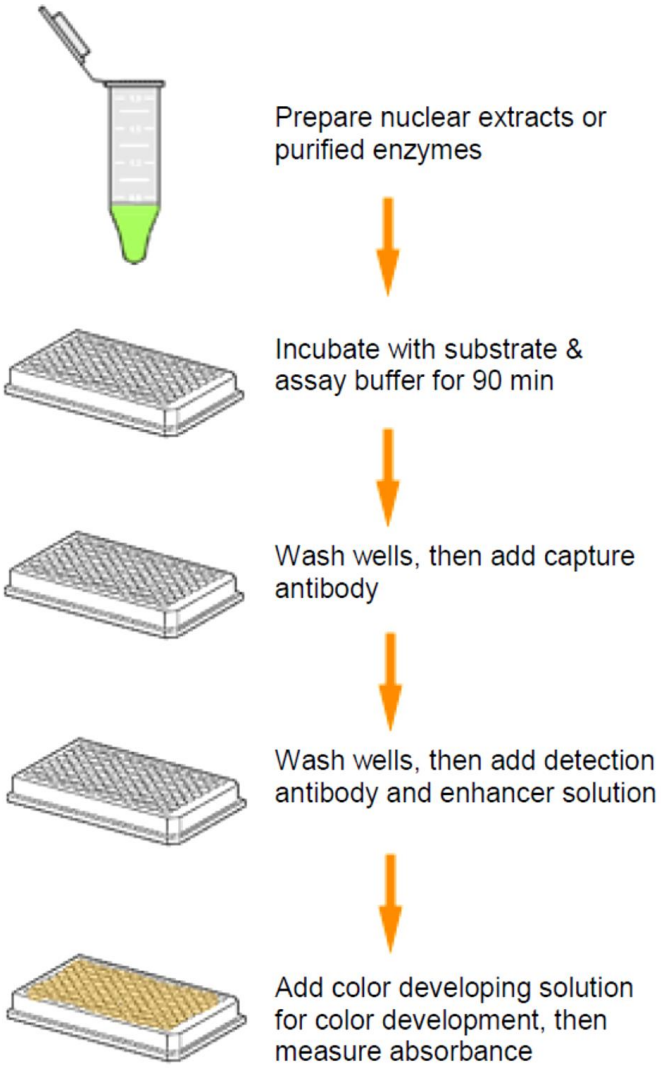
The importance of DNA methylation is emphasized by the growing number of human diseases that are known to occur when DNA methylation information is not properly established and/or maintained. Abnormal DNA methylation associated with increased expression or the activity of DNMTs has been found in many 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. The DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric) addressed this issue by introducing a simple method with an ELISA-like 96-well plate format. This kit enhances sample signals and significantly minimizes background signals, in addition to being more sensitive.

- Colorimetric 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 colorometric assay without radioactivity, extraction, and chromatography.
- The ultra-sensitive detection limit can be as low as 0.5 µg of nuclear extract or 0.5 ng of purified enzymes.
- Optimized antibody and 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.

Principle of the Assay

The DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric) 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 a 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 through an ELISA-like reaction by reading the absorbance in a microplate spectrophotometer at a wavelength of 450 nm. The activity of DNMT enzymes is proportional to the optical density intensity measured.



Schematic procedure of DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric)

General Information

Materials Supplied

List of component

Component	Amount	Storage Upon Receipt
MU1 (10X Wash Buffer)	14 mL	4°C
MU2 (DNMT Assay Buffer)	4 mL	RT
MU3 (Adomet, 50X)*	60 µL	-20°C
MU4 (DNMT Enzyme Control, 50 µg/mL)*	6 µL	-20°C
MU5 (Capture Antibody, 1000 µg/mL*)	5 µL	4°C
MU6 (Detection Antibody, 400 µg/mL)*	6 µL	-20°C
MU7 (Enhancer Solution)*	6 µL	-20°C
MU8 (Developer Solution)	5 mL	4°C
MU9 (Stop Solution)	5 mL	RT
8-Well Assay Strips (With Frame)	6 strips	4°C
Adhesive Covering Film	1 slice	RT

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

Storage Instruction

Upon receipt: (1) Store MU3, MU4, MU6, and MU7 at -20°C away from light; (2) Store MU1, MU5, MU8, and the 8-Well Assay Strips at 4°C away from light; (3) Store all remaining components (MU2, MU9, and the Adhesive Covering Film) at room temperature away from light.
All components of the kit are stable for 6 months from the date of shipment, when stored properly.

Note: (1) Check if MU1 (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; and (2) transfer the amount of MU8 required into a secondary container (tube or vial) before adding MU8 into the assay wells in order to avoid contamination. Check if a blue color is present in MU8 (Developer Solution) before each use, as this would indicate contamination of the solution and should not be used.

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 enzyme samples containing DNMT activity
- ✓ Dnmt inhibitors (optional)
- ✓ Parafilm M or aluminium foil

Precautions for Use

- ✓ Input Material: Input materials can be nuclear extracts or purified DNMT enzymes, the amount of nuclear extracts for each assay can be between 0.5 µg to 20 µg with an optimal range of 5-10 µg. The amount of purified enzymes can be 0.5 ng to 200 ng, depending on the quality and catalytic activity of the enzymes.
- ✓ Internal Control: A positive enzyme control is provided in this kit. Because DNMT activity can vary from tissue to tissue, and from normal and abnormal 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.

- ✓ General Product Information
- Quality control: Each lot of this product is tested against predetermined specifications to ensure consistent product quality. Abnova 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: Abnova 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. Thus, only use the User Guide that was supplied with the kit when using that kit.
- Usage Limitation: The DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric) is for research use only and is not intended for diagnostic or therapeutic applications.
- Intellectual Property: The DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric) and methods of use contain proprietary technologies by Abnova.

Assay Protocol

Reagent Preparation

1. Prepare Diluted MU1 1X Wash Buffer: Add 13 mL of MU1 (10X Wash Buffer) to 117 mL of distilled water and adjust pH to 7.2-7.5.
   This Diluted MU1 1X Wash Buffer can now be stored at 4°C for up to six months.
2. Prepare Diluted MU3 Working Buffer: Freshly prepare the Diluted MU3 Working Buffer required for the assay by adding 2 µL of MU3 into 98 µL of MU2 (DNMT Assay Buffer). About 50 µL of this Diluted MU3 will be required for each assay well.
3. Prepare Diluted MU5 Capture Antibody Solution: Dilute MU5 (Capture Antibody) with Diluted MU1 at a ratio of 1:1000 (i.e., add 1 µL of MU5 to 1000 µL of Diluted MU1). About 50 µL of this Diluted MU5 will be required for each assay well.
4. Prepare Diluted MU6 Detection Antibody Solution: Dilute MU6 (Detection Antibody) with Diluted MU1 at a ratio of 1:2000 (i.e., add 1 µL of MU6 to 2000 µL of Diluted MU1). About 50 µL of this Diluted MU6 will be required for each assay well.
5. Prepare Diluted MU7 Enhancer Solution: Dilute MU7 Enhancer Solution with Diluted MU1 at a ratio of 1:5000 (i.e., add 1 µL of MU7 to 5000 µL of Diluted MU1). About 50 µL of this Diluted MU7 will be required for each assay well.
6. About the MU4 DNMT Enzyme Control: The MU4 (DNMT Enzyme Control) is an enzyme with activity of both maintenance and *de novo* DNMTs and is used as the positive control of this 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.
   Note: Keep each of the diluted solutions (except Diluted MU1 1X Wash Buffer) on ice until use. Any remaining diluted solutions, other than Diluted MU1, should be discarded if not used within the same day.

Sample Preparation

Starting Materials

- ✓ 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 to 10 µg. The amount of purified enzymes can be 0.5 ng to 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. We also offer a Nuclear Extraction Kit (Cat # KA6124) optimized for use with this kit.
- ✓ Nuclear Extract or Purified DNMT Storage: Nuclear extract or purified DNMT enzymes should be stored at -80°C until use.

Assay Procedure

A. Enzymatic Reaction

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).
2. Blank Wells: Add 50 µL of Diluted MU3 per well.
3. Positive Control Wells: Add 50 µL of Diluted MU3 and 1 µL of MU4 per well.
4. Sample Wells Without Inhibitor: Add 45 µL to 49 µL of Diluted MU3, and 1 µL to 5 µL of nuclear extracts or 1 to 5 µL of purified DNMT enzymes per well. The total volume should be 50 µL per well.
5. Sample Wells With Inhibitor: Add 40 µL to 44 µL of Diluted MU3, 1 to 5 µL of nuclear extracts or 1 to 5 µL of purified DNMT enzymes, and 5 µL of inhibitor solution per well. The total volume should be 50 µL per well.

   Note: (1) Follow suggested well setup diagrams; (2) It is recommended to use 5 µg to 10 µg of nuclear extract per well or 10 ng to 100 ng of purified enzyme per well; (3) The concentration of inhibitors to be added into the sample wells can be varied (e.g., 1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with MU2 at a 1:10 ratio (e.g., add 0.5 µL of inhibitor to 4.5 µL of MU2), so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less.
6. Tightly cover the strip-well microplate with Adhesive Covering Film to avoid evaporation, and incubate at 37°C for 90-120 min.

   Note: (1) 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; (2) The Adhesive Covering Film can be cut to the required size to cover the strips based on the number of strips to be used.
7. Remove the reaction solution from each well. Wash each well three times with 150 µL of the Diluted MU1 1X Wash Buffer each time. This can be done by simply pipetting Diluted MU1 in and out of the wells.

B. Antibody Binding and Signal Enhancing

1. Add 50 µL of the Diluted MU5 to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 60 min.
2. Remove the Diluted MU5 solution from each well.
3. Wash each well with 150 µL of the Diluted MU1 each time for three times.
4. Add 50 µL of the Diluted MU6 to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.
5. Remove the Diluted MU6 solution from each well.
6. Wash each well with 150 µL of the Diluted MU1 each time for four times.

7. Add 50 µL of the Diluted MU7 to each well, then carefully cover with Parafilm M or aluminium foil and incubate at room temperature for 30 min.
8. Remove the Diluted MU7 solution from each well.
9. Wash each well with 150 µL of the Diluted MU1 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.

C. Signal Detection

1. Add 100 µL of MU8 to each well and incubate at room temperature for 1 to 10 min away from direct light. Monitor color change in the sample wells and control wells. The MU8 solution will turn blue in the presence of sufficient methylated DNA.
2. Add 100 µL of MU9 to each well to stop enzyme reaction when the color in the positive control wells turns medium blue. Mix the solution by gently shaking the frame and wait 1-2 min to allow the color reaction to be completely stopped. The color will change to yellow after adding MU9 and 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 the capability to carry out dual wavelength analysis and will automatically subtract the 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 manualy 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.

Data Analysis

Calculation of Results

- DNMT Activity Calculation

1. Calculate the average duplicate readings for sample wells and blank wells.
2. Calculate DNMT activity using the following formula:

$$\text{DNMT Activity (OD/h/mg)} = \frac{\text{(Sample OD – Blank OD)}}{\text{(Protein amount (µg)* x hour**)}} \times 1000$$

* Protein amount added into the reaction at step A4 in µg.

** Incubation time at step A6.

Example calculation:

Average OD450 of sample is 0.55

Average OD450 of blank is 0.05

Protein amount is 5 µg

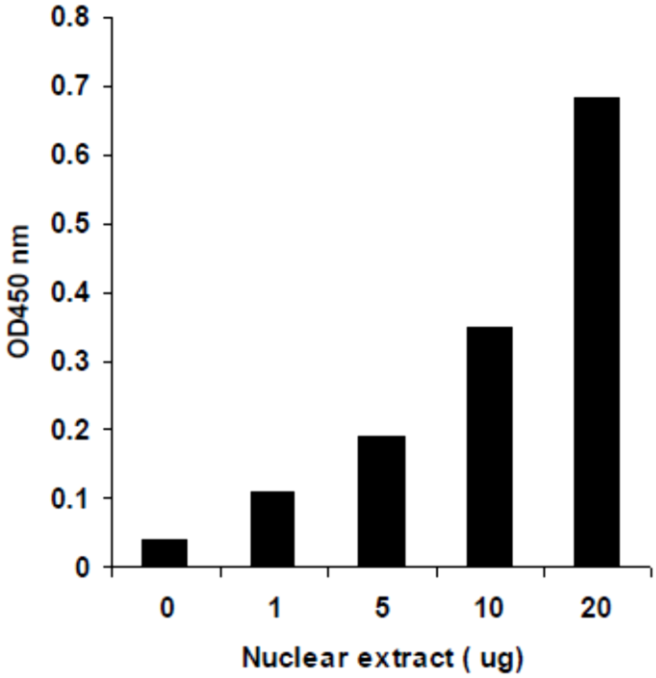
Incubation time is 2 hours (120 min)

$$\text{DNMT Activity} = \frac{(0.55 - 0.05)}{(5 \times 2)} \times 1000 = 50 \text{ OD/h/mg}$$

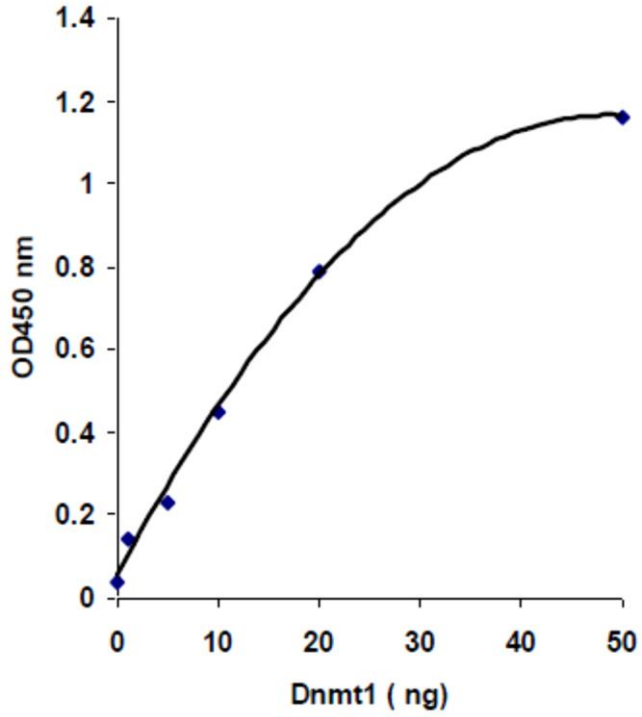
3. Calculate DNMT inhibition using the following formula:

$$\text{DNMT Inhibition \%} = \left[1 - \frac{\text{Inhibitor Sample OD – Blank OD}}{\text{No Inhibitor Sample OD – Blank OD}} \right] \times 100\%$$

Performance Characteristics



Demonstration of high sensitivity and specificity of the DNMT activity assay achieved by using nuclear extracts with the DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric).



Demonstration of high sensitivity and specificity of the DNMT activity/inhibition assay achieved by using recombinant DNMT1 with DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric).

Resources

Troubleshooting

Problem	Possible Cause	Suggestion
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 standard control and sample.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Incorrect absorbance reading.	Check if 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 temperature and the cap is tightly capped after each opening or use.
No signal or weak signal in only the positive control wells	The DNMT enzyme control is insufficiently added to the well in Step A3.	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 Instruction of this protocol for MU4 (DNMT Enzyme Control).
High background present in the blank wells	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 B4 should not exceed 45 min.
	Over development of color.	Decrease the development time in Step C1 before adding MU9 (Stop Solution) in Step C2.
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. For the best results, it is advised to use the Nuclear Extraction Kit (Cat # KA6124). 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 step " Enzymatic Reaction". 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.
	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 color 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 color development or delayed stopping of color development in the wells.	Ensure color 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).
Large variation between replicate wells	Color reaction is not evenly stopped due to an inconsistency in pipetting time.	Ensure MU8 Developer Solution and MU9 Stop Solution are added at the same time between replicates or otherwise maintain a consistent timing in between each addition of solutions.
	Color reaction is not evenly stopped due to an inconsistency order of adding solutions.	Ensure all solutions, particularly MU8 Developer Solution and MU9 Stop Solution, are added in the same order each time as all other solutions.
	The solutions are not evenly added due to inconsistency in pipetting volume.	Ensure the solution in each pipette tip is equal in the multi-channel pipette. Equilibrate the pipette tip in any solutions before adding them. Ensure the solutions, especially those with small volumes (e.g. 1 µL) are completely added into the wells.
	Solutions or antibodies were not actually added into the wells.	Do not allow pipette tip to touch the outer edges or inner sides of the wells to prevent solutions from sticking to the surface.
	Did not sufficiently shake the solutions in the wells evenly after adding MU9 Stop Solution in Step	Gently and evenly shake the plate frame across a flat surface so that the solutions in the wells are better distributed. Do not stir.

	C2.	
	Did not use the sample pipette device throughout the experiment.	Use the same multi-channel pipette device throughout the entire experiment, as different pipette devices may have slight variations in performance.
Capture Antibody vial appears to be empty or insufficient in volume	Buffer evaporated due to the very small volumes, resulting in a higher concentrated antibody.	Add 1X PBS buffer into the Capture Antibody vial until you restore the correct, intended volume according to the Kit Contents described in this protocol. Mix and centrifuge prior to use.

Suggested Working Buffer and solution Setup

Reagents	1 well	8 wells	16 wells	48 wells
		(1 strip)	(2 strips)	(6 strips)
Diluted MU1	2.5 mL	20 mL	40 mL	120 mL
Diluted MU3	50 µL	400 µL	800 µL	2400 µL
Diluted MU5	50 µL	400 µL	800 µL	2400 µL
Diluted MU6	50 µL	400 µL	800 µL	2400 µL
Diluted MU7	50 µL	400 µL	800 µL	2400 µL
Developer Solution	0.1 mL	0.8 mL	1.6 mL	4.8 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

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

Plate Layout

	1	2	3	4	5	6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	MU4 0.5 µL	MU4 0.5 µL	Sample	Sample	Sample	Sample
C	MU4 1 µL	MU4 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

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.