# DNMT Activity / Inhibition Assay

(version B2)

Catalog No. 55006

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## Overview

DNA methylation is a major epigenetic modification in the genome of higher eukaryotes. This DNA covalent modification is catalyzed by DNA methyltransferase enzymes (DNMTs) and consists in the addition of a methyl group from S-adenosyl-L-methionine (AdoMet) to the fifth carbon position of cytosine (C5), mostly within CpG dinucleotides. This methylation, together with histone modifications, plays an important role in modulating chromatin structure, thus controlling gene expression and many other chromatin-dependent processes.

Active Motif's DNMT Activity / Inhibition Assay is a time-saving, non-radioactive assay to measure DNA methyltransferase activity and/or inhibition from recombinant DNMT enzymes (DNMT1, DNMT3a & DNMT3b) or nuclear extract samples. This sensitive ELISA-based method uses the ability of methyl CpG binding domain (MBD) proteins to bind methylated DNA with high affinity. In the DNMT assay method, a universal CpG-enriched DNA substrate has been immobilized on a 96-stripwell plate. Purified DNMTs or DNMT activities from nuclear extracts will catalyze the transfer of methyl groups from the provided AdoMet reagent to the coated DNA substrate. The resulting methylated DNA will be recognized by the His-tagged recombinant MBD2b in an amount proportional to the enzyme activity. Addition of a polyHistidine antibody conjugated to horseradish peroxidase (HRP) provides a sensitive colorimetric readout that is easily quantified by spectrophotometry. The assay includes a CpG methyltransferase enzyme as a positive control.

| product | format | catalog no. |
|---|---|---|
| DNMT Activity / Inhibition Assay | 1 x 96 rxns | 55006 |

## Introduction

**DNMT Activity / Inhibition Assay**

One of the earliest identified forms of epigenetic regulation of gene expression is DNA methylation. In vertebrates, DNA methylation occurs predominantly on the CpG dinucleotide and approximately 60 to 90% of these dinucleotides are modified. Distinct DNA methylation patterns, which can vary between different tissues and developmental stages, exist on specific loci. Since CpG sequences are frequently clustered in proximal promoters of genes in higher eukaryotes, modification at these sequences can have profound effects on transcription. DNA methylation is associated with inhibition of transcription and a repressed chromatin state[1,2], as well as X chromosome inactivation[3,4] and maintenance of imprinted genes[5]. DNA methylation can cause gene silencing by interference with recognition and binding of positively acting transcription factors or by promotion of interaction and recruitment of negative factors. These last factors include methyl-CpG-binding domain (MBD) proteins which recruit the enzymatic machinery to establish silent chromatin[6].

In mammals and other vertebrates, DNA methylation occurs at the C5 position of cytosine (5mC), mostly within CpG dinucleotides. In this process, the enzymes use a conserved mechanism that has been best studied in the bacterial 5mC methyltransferase, M.HhaI[7,8]. Briefly, the target cytosine is flipped out of the double helix (base flipping) and the recipient C5 position is activated by a transient, covalent complex formation with the enzyme at the C6 position. After methyl group transfer, the enzyme is released by beta-elimination together with the proton at position C5.

DNA methylation is accomplished by three enzymes: DNMT1, DNMT3a and DNMT3b. DNMT3a and DNMT3b fall in the group of *de novo* methyltransferases (MTases)[9], enzymes that are able to methylate previously unmethylated CpG sequences, while DNMT1 functions as a maintenance methylase, copying pre-existing methylation marks onto the new DNA strand during replication[10]. Although generally thought of as a maintenance enzyme that acts on hemimethylated DNA, DNMT1 has also been shown to function as a *de novo* DNA methyltransferase[11,12,13]. In addition, two non-canonical family members, DNMT2 and DNMT3L, have been discovered[14,15]: DNMT2 methylates cytosine 38 in the anticodon loop of tRNA16, while DNMT3L is a catalytically inactive DNMT. DNMT3L is known to associate with both DNMT3a and DNMT3b and may be responsible for the recruitment of histone deacetylases to direct repression onto newly established imprints[17]. It is still not clear how methyl groups are removed from DNA. Several studies recently suggest that active DNA demethylation might be accomplished through a process of DNA repair[18,19] that involves nucleotide exchange[20,21], replacing 5-methylcytosine with unmodified cytosine. This could be the physiological mechanism that operated during normal development *in vivo*[22].

Although DNA methylation and histone modification are carried out by different chemical reactions and require different sets of enzymes, there seems to be a biological relationship between the two systems that plays a part in modulating gene repression programming in the organism[23,24]. This relationship between DNA methylation and histone modification might be partially mediated through methylcytosine-binding proteins, such as MECP2 or MBD2, that are capable of recruiting histone deacetylases to the methylated regions[25,26]. It is probable that the presence of DNA methylation also directs H3K9 dimethylation, which is a mark of repressive chromatin, perhaps through the interaction of G9a and DNMT1 with the replication complex[27]. There is also

evidence that DNA methylation inhibits H3K4 methylation[28,29] and, in plants, excludes the histone variant H2AZ from nucleosomes[30]; both of these marks are associated with active transcription.

Understanding the relationship between DNA methylation and certain histone modifications is also providing insights into the aberrant gene expression patterns observed in cancer[31]. *De novo* DNA methylation plays a critical role in the development of certain cancers, *e.g.* renal cell carcinoma, colon carcinoma, lung cancer and many others. More specifically, hypermethylation of tumor suppressor genes is one of the most consistent hallmarks of human cancers. DNA methyltransferase inhibitors, therefore, represent promising new drugs for cancer therapies[32,33,34]. Some of these compounds (nucleoside analogs or DNMT1 antisense molecules) have recently been approved as antitumor agents and others are presently in various stages of their pre-clinical or clinical development[35,36].

## Traditional Methods to Study DNA Methyltransferase Activity

To date, there are several methods used to study DNA methyltransferase activity / inhibition.

1. **Radioactively labeled AdoMet:** In this method the methyltransferase activity is measured through the transfer of radioactively labeled methyl groups from the cofactor AdoMet to DNA substrates[37,38,39,40].
2. **Methylation-sensitive restriction enzyme analysis:** Isoschizomers of bacterial restriction endonucleases with different sensitivities for 5-methylcytosine can be used to determine the methylation status of specific CpG-dinucleotides[41]. Methylation-sensitive restriction enzymes have several limitations including that methylation-sensitive restriction merely informs on the methylation status of the cytosine residues which are recognized by the restriction enzymes used.
3. **Mass spectrometry:** Direct detection of methylated cytosine residues can be accomplished by MALDI-TOF mass spectrometry[42], or by monitoring the conversion of AdoMet to S-adenosyl-homocysteine by liquid chromatography and mass spectrometry[43].

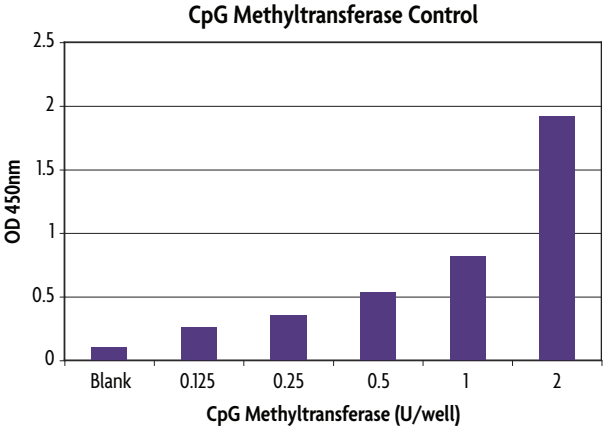
Active Motif's DNMT Activity / Inhibition Assay is a fast, user-friendly assay to specifically detect DNA methyltransferase activity from recombinant DNMT enzymes or nuclear extract samples without the need for radioisotopes or expensive equipment. This innovative method utilizes the ability of methyl CpG binding domain (MBD) proteins to bind methylated DNA with high affinity. A universal CpG-enriched DNA substrate has been immobilized in the wells of a 96-stripwell plate. Purified DNMTs or DNMT activities from nuclear extracts will catalyze the transfer of methyl groups from the provided AdoMet reagent to the coated DNA substrate. The resulting methylated DNA will be recognized by the His-tagged recombinant MBD2b in an amount proportional to the enzyme activity. Addition of a polyHistidine antibody conjugated to horseradish peroxidase (HRP) provides a sensitive colorimetric readout that is easily quantified by spectrophotometry.

## Kit Performance and Benefits

**Range of detection:** This assay is able to detect DNMT activity from purified enzymes in the range of 0.5 to 100 ng per well. For nuclear extract samples, we recommend using 0.5 to 10 µg per well. The assay is able to detect DNMT1, DNMT3a and DNMT3b activity, but will not distinguish between the different enzymes. In order to detect activity of a specific DNMT, we recommend using purified DNMT of the isoform of interest.

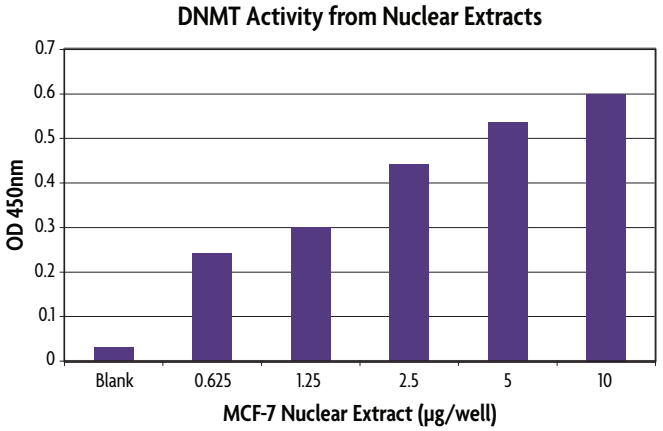
**Assay time:** 3 hours.

## DNMT Activity / Inhibition Assay



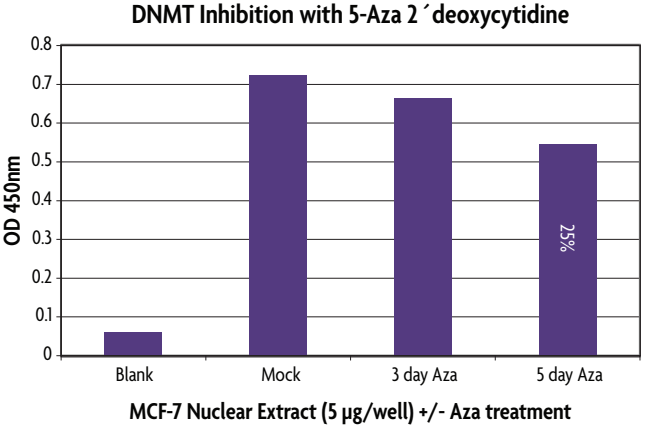

**DNMT activity using a CpG Methyltransferase.**

The DNMT Activity / Inhibition Assay was used to generate a standard curve using the provided CpG Methyltransferase control enzyme. The assay was able to detect DNMT activity from as little as 0.125 units of enzyme with a 1.5 hour incubation and a 5 minute developing time. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.




**DNMT activity from MCF-7 nuclear extracts.**

The DNMT Activity / Inhibition Assay was used to screen MCF-7 nuclear extracts (0.625 - 10 µg) prepared using Active Motif's Nuclear Extract Kit (Catalog No. 40010). The assay was able to detect DNMT activity from as little as 0.625 µg of extract with a 1.5 hour incubation and a 5 minute developing time. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.




**DNMT inhibition with 5-Aza 2´deoxycytidine treatment.**

The DNMT Activity / Inhibition Assay was used to screen for DNMT inhibition in MCF-7 cells that were either untreated, or treated with 5-Aza 2´deoxycytidine for 3 - 5 days. Nuclear extracts were prepared using Active Motif's Nuclear Extract Kit (Catalog No. 40010) and 5 µg of each treatment condition was tested in the assay with a 1.5 hour incubation time and 3 minute developing time. The 5-Aza 2´deoxycytidine treated extracts showed a 25% inhibition of DNMT activity as compared to the mock treated sample for 5 day treatment. Data shown are the results from wells assayed in duplicate. These results are provided for demonstration only.

## Kit Components and Storage

DNMT Activity / Inhibition Assay Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

| Reagents | Quantity | Storage |
|---|---|---|
| Anti-polyHis-HRP antibody | 11 µl | -20°C |
| Enzymatic Buffer AM1 | 11 ml | -20°C |
| 100X AdoMet | 110 µl | -20°C |
| CpG Methyltransferase Enzyme (2 U/µl) | 8 µl | -20°C |
| His-MBD2b protein | 220 µl | -20°C |
| Binding Buffer AM11 | 11 ml | 4°C |
| 10X Wash Buffer AM1 | 15 ml | 4°C |
| 10X Wash Buffer AM3 | 7 ml | 4°C |
| 10X Antibody Binding Buffer AM3 | 1.5 ml | 4°C |
| Developing Solution | 11 ml | 4°C |
| Stop Solution | 11 ml | 4°C |
| 96-well Assay Plate | 1 | 4°C |
| Plate sealer | 1 | RT |

**Additional materials required**

- Recombinant DNMT enzyme or nuclear extract samples containing DNMT activity. For your convenience Active Motif also offers a Nuclear Extract Kit which can be used for the preparation of nuclear extract samples (Cat. Nos. 40010 & 40410).
- Multi-channel pipettor
- Multi-channel pipettor reservoirs
- Microcentrifuge tubes
- Rocking platform at RT
- Rocking platform at 37°C
- Microplate spectrophotometer capable of reading at 450 nm (655 nm as optional reference wavelength)

## Buffer Preparation and Recommendations

**Preparation of Complete Enzymatic Buffer**

Prepare the amount of Complete Enzymatic Buffer required for the assay by adding 1 µl of 100X AdoMet per 99 µl of Enzymatic Buffer AM1 and keep on ice (see the Quick Chart for Preparing Buffers in this section). We recommend adding AdoMet immediately prior to use. Any remaining Complete Enzymatic Buffer should be discarded if not used in the same day.

**Note:** The Enzymatic Buffer does contain EDTA. Therefore, researchers interested in studying metal co-activators should use an alternate buffer as suggested in the Troubleshooting Guide in Section A of the Appendix.

**Preparation of 1X Wash Buffer AM1**

Prepare the amount of 1X Wash Buffer AM1 required for the assay as follows: For every 10 ml of 1X Wash Buffer AM1 required, dilute 1 ml 10X Wash Buffer AM1 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer AM1 may be stored at 4°C for one week. The Tween 20 contained in the 10X Wash Buffer AM1 may form clumps, therefore it is necessary to completely resuspend any precipitates by incubating at 50°C for 2 minutes and mixing prior to use.

**Preparation of His-MBD2b Dilution**

Prepare the amount of His-MBD2b required for the assay by adding 2 µl of His-MBD2b protein per 98 µl of Binding Buffer AM11 and mix thoroughly (see the Quick Chart for Preparing Buffers in this section). We recommend diluting His-MBD2b protein immediately prior to use. Any remaining His-MBD2b dilution should be discarded if not used in the same day. During the first use, we recommend making small aliquots of the His-MBD2b stock protein and storing at -20°C to avoid multiple freeze/thaw cycles.

**Preparation of 1X Wash Buffer AM3**

Prepare the amount of 1X Wash Buffer AM3 required for the assay as follows: For every 10 ml of 1X Wash Buffer AM3 required, dilute 1 ml 10X Wash Buffer AM3 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The 1X Wash Buffer AM3 may be stored at 4°C for one week. The BSA contained in the 10X Wash Buffer AM3 may form clumps, therefore it is necessary to completely resuspend any precipitates by incubating at 37°C for 2 minutes and mixing prior to use.

**Preparation of 1X Antibody Binding Buffer AM3**

Prepare the amount of 1X Antibody Buffer AM3 required for the assay as follows: For every 10 ml of 1X Antibody Binding Buffer AM3 required, dilute 1 ml 10X Antibody Binding Buffer AM3 with 9 ml distilled water (see the Quick Chart for Preparing Buffers in this section). Mix gently to avoid foaming. The BSA contained in the 10X Antibody Binding Buffer AM3 may form clumps, therefore it is necessary to completely resuspend any precipitates by incubating at 37°C for 2 minutes and mixing prior to use. Discard remaining 1X Antibody Binding Buffer AM3 after use.

### Preparation of Anti-polyHis-HRP antibody

Dilute the anti-polyHis-HRP antibody 1:1000 with 1X Antibody Binding Buffer AM3 and mix thoroughly. Use 100 µl per well.

### Developing Solution

The Developing Solution should be warmed to room temperature before use. The Developing Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. However, a blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting into the wells (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Developing Solution.

### Stop Solution

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard remaining Stop Solution.

**WARNING:** The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* safety glasses, gloves and labcoat.

### CpG Methyltransferase enzyme

A small volume of CpG Methyltransferase enzyme is provided as a positive control for the assay. **Please centrifuge the enzyme vial before opening the cap to ensure complete recovery of the small volume of material inside the vial.** There is enough positive control enzyme included in the kit to generate two standard curves. The CpG Methyltransferase is provided at 2 U/µl.

### Preparation of DNMT containing samples

Purified DNMT enzymes or nuclear extracts containing DNMT activity can be used in the assay. For purified DNMT enzymes a range of 0.5 ng - 100 ng is recommended, while for nuclear extracts a range of 0.5 µg - 10 µg is suggested. It is recommended initially to use a range of sample concentrations in order to determine the amount of sample necessary to fall within the linear range of the assay. Once the appropriate amount of sample has been determined, perform the rest of the assays within the linear range. It is recommended to work with fresh DNMT or nuclear extract samples. If samples need to be stored, small aliquots should be prepared and stored at -80°C to avoid multiple freeze/thaw cycles. To prepare nuclear extracts, we recommend using Active Motif's Nuclear Extract Kit (Catalog No. 40010). The use of fresh cell or tissue samples for extract preparation is suggested, as frozen samples can lose enzymatic activity.

## Quick Chart for Preparing Buffers

| Reagents to prepare | Components | For 1 well | For 1 strip (8 wells) | For 6 strips (48 wells) | For 12 strips (96 wells) |
|---|---|---|---|---|---|
| Complete Enzymatic Buffer AM1 | Enzymatic Buffer AM1 | 109 µl | 891 µl | 5.35 ml | 10.7 ml |
| | 100X AdoMet | 1.1 µl | 9 µl | 54 µl | 108 µl |
| | **TOTAL REQUIRED** | **110 µl** | **900 µl** | **5.4 ml** | **10.8 ml** |
| 1X Wash Buffer AM1 | Distilled water | 1.35 ml | 10.8 ml | 63 ml | 117 ml |
| | 10X Wash Buffer AM1 | 150 µl | 1.2 ml | 7 ml | 13 ml |
| | **TOTAL REQUIRED** | **1.5 ml** | **12 ml** | **70 ml** | **130 ml** |
| His-MBD2b Dilution | Binding Buffer AM11 | 107.8 µl | 882 µl | 5.29 ml | 10.58 ml |
| | His-MBD2b protein | 2.2 µl | 18 µl | 108 µl | 216 µl |
| | **TOTAL REQUIRED** | **110 µl** | **900 µl** | **5.4 ml** | **10.8 ml** |
| 1X Wash Buffer AM3 | Distilled water | 630 µl | 4.95 ml | 29.7 ml | 58.5 ml |
| | 10X Wash Buffer AM3 | 70 µl | 550 µl | 3.3 ml | 6.5 ml |
| | **TOTAL REQUIRED** | **700 µl** | **5.5 ml** | **33 ml** | **65 ml** |
| 1X Antibody Binding Buffer AM3 | Distilled water | 99 µl | 810 µl | 4.86 ml | 9.72 ml |
| | 10X Antibody Binding Buffer AM3 | 11 µl | 90 µl | 540 µl | 1.08 ml |
| | **TOTAL REQUIRED** | **110 µl** | **900 µl** | **5.4 ml** | **10.8 ml** |
| Anti-polyHis-HRP Ab | 1X Antibody Binding Buffer AM3 | 110 µl | 900 µl | 5.4 ml | 10.8 ml |
| | Anti-polyHis-HRP Ab | 0.11 µl | 0.9 µl | 5.4 µl | 10.8 µl |
| | **TOTAL REQUIRED** | **110 µl** | **900 µl** | **5.4 ml** | **10.8 ml** |
| Developing Solution | **TOTAL REQUIRED** | **110 µl** | **900 µl** | **5.4 ml** | **10.8 ml** |
| Stop Solution | **TOTAL REQUIRED** | **110 µl** | **900 µl** | **5.4 ml** | **10.8 ml** |

## Read the entire protocol before use.

Determine the appropriate number of microwell strips required for testing samples, controls and blanks in duplicate. Store the unused strips in the aluminum pouch at 4°C. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the plate sealer while you perform the assay. The unused wells are stable at room temperature for the duration of the assay if kept dry. Once the assay is finished, unused strips should be returned to the aluminum pouch and stored at 4°C for a separate assay. Use the strip holder while performing the assay.

Prepare all the buffers required as described above in the section Buffer Preparation and Recommendations. Multi-channel pipettor reservoirs may be used for dispensing the 1X Wash Buffer AM1, His-MBD2b dilution, 1X Wash Buffer AM3, Anti-polyHis-HRP antibody dilution, Developing Solution and Stop Solution into the wells being used.

All of the washes in the DNMT assay protocol can be performed by inverting the plate and "flicking" the fluid into a sink. The inverted plate is then tapped on clean absorbent paper to remove excess fluid.

## Standard Curve Preparation (Optional)

Use this plate set-up example to prepare a standard curve for the included CpG Methyltransferase enzyme in duplicate. The positive control CpG Methyltransferase is tested from 0.125 - 2 U per well in a 2-fold serial dilution. Prepare 1.5 ml Complete Enzymatic Buffer for use in the standard curve by adding 15 µl 100X AdoMet to 1.485 ml Enzymatic Buffer. Vortex to mix.

| | Positive Control | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| A | 2 U | 2 U | – | – | – | – | – | – | – | – | – | – |
| B | 1 U | 1 U | – | – | – | – | – | – | – | – | – | – |
| C | 0.5 U | 0.5 U | – | – | – | – | – | – | – | – | – | – |
| D | 0.25 U | 0.25 U | – | – | – | – | – | – | – | – | – | – |
| E | 0.125 U | 0.125 U | – | – | – | – | – | – | – | – | – | – |
| F | Blank | Blank | – | – | – | – | – | – | – | – | – | – |
| G | – | – | – | – | – | – | – | – | – | – | – | – |
| H | – | – | – | – | – | – | – | – | – | – | – | – |

1. The CpG Methyltransferase enzyme is provided at a 2 U/µl concentration. Keep the enzyme on ice. Before using, quick spin the contents to the bottom of the tube.
2. Add 200 µl of Complete Enzymatic Buffer to wells A1 and A2.

3. Add 100 µl of Complete Enzymatic Buffer to wells B1 through F2.
4. Add 2 µl CpG Methyltransferase enzyme to wells A1 and A2. Pipet up and down to mix.
4. Perform a two-fold serial dilution by transferring 100 µl of the contents in row A to the wells in row B.
5. Mix the contents of row B by pipetting up and down 3-5 times. Do not change pipette tips between well transfers.
6. Transfer 100 µl of the contents of row B to row C and mix, as previously described.
7. Continue this process until row E is reached.
8. When row E is reached, discard 100 µl of the well contents so that the final volume is 100 µl.
9. Row F will serve as the blank wells.

## Step 1: Enzymatic Reaction

1. In duplicate, prepare sample reactions. It is recommended to initially try a range of concentrations in order to determine the amount of sample needed to fall within the linear range of the assay. Follow the chart below to add sample, optional co-activator/inhibitor and complete Enzymatic Buffer to reach a final volume of 100 µl per well.

   **Purified DNMT enzymes:** Recommended range of 0.5 ng - 100 ng per well

   **Nuclear extracts:** Recommended range of 0.5 µg - 10 µg per well

   **Blank Wells:** Add 100 µl Complete Enzymatic Buffer to each well

| Reagent | One Reaction (Double amounts for duplicates) |
|---|---|
| Purified DNMT or Nuclear Extract | See above recommendations |
| Co-activator or Inhibitor | X µl |
| Complete Enzymatic Buffer | Up to 100 µl final volume |

2. Using the provided adhesive plate sealer, cover the plate. Incubate the plate containing the protein standard curve (optional) and samples for 1-2 hour(s) at 37°C with mild agitation (100 rpm on a rocking platform). The incubation time depends on the intrinsic DNMT enzymatic activity of your samples, but some guidelines are suggested below:

   **1 hour incubation:** Recommended for active DNMT1 enzymes and optional standard curve

   **2 hour incubation:** Recommended for active DNMT3a/3b enzymes and nuclear extracts
3. After the incubation, remove the binding reaction with a multichannel pipette. Use clean tips for each well to avoid cross-contamination of samples.
4. Wash the wells 3 times with 200 µl of 1X Wash Buffer **AM1**.

## Step 2: Binding of His-MBD2b protein

5. Add 100 µl of diluted His-MBD2b protein (1:50 dilution in Binding Buffer AM11) to all of the wells.
6. Incubate at room temperature for 45 minutes with mild agitation (100 rpm on a rocking platform).
7. After the incubation, wash the wells 3 times with 200 µl of 1X Wash Buffer **AM3**.

## Step 3: Binding of poly-Histidine HRP Antibody

8. Add 100 µl of diluted anti-polyHis-HRP antibody (1:1000 dilution in 1X Antibody Binding Buffer AM3) to all of the wells.
9. Incubate at room temperature for 45 minutes with mild agitation (100 rpm on a rocking platform).
10. During this incubation, place the Developing Solution at room temperature.
11. After the incubation, wash the wells 3 times with 200 µl of 1X Wash Buffer **AM1**.

## Step 4: Colorimetric Reaction

12. Remove as much of the final wash as possible by blotting the plate on paper towels.
13. Add 100 µl of room temperature Developing Solution to all wells being used.
14. Incubate under low light conditions from 30 seconds to 10 minutes at room temperature protected from direct light. Monitor the blue color development in the wells containing the higher concentrations of sample until they turn medium to dark blue. Do not overdevelop. Please read the Certificate of Analysis supplied with this kit for optimal development time associated with the CpG Methyltransferase standard curve.
15. Add 100 µl of Stop Solution to all the wells. In presence of the acid, the blue color turns yellow.
16. Read absorbance on a spectrophotometer within 5 minutes at 450nm with an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

    Reading the reference wavelength is optional. Most microtiter plate readers are equipped to perform dual wavelength analysis and with the appropriate software, will automatically subtract the reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, you may read the plate twice, once at 450 nm and once at 655 nm then manually subtract the 655 nm OD from the 450 nm OD values.

**Calculation of DNMT Activity or Inhibition**

To calculate activity or inhibition of DNMTs, average the duplicate readings for sample and blank wells and follow the formulas below:

$$\textbf{DNMT activity}\ (\text{OD/h/mg}) = \frac{(\text{Average Sample OD} - \text{Average Blank OD})}{\text{Protein amount } (\mu\text{g})^{*} \times \text{hour}^{**}} \times 1000$$

*Protein amount added into the reaction in Step 1.1

**Incubation time used for the reaction in Step 1.2

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

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# Appendix

## Section A: Troubleshooting Guide

| Problem/question | Possible cause | Recommendation |
|---|---|---|
| No signal or weak signal | Omission of key reagent | Check that all reagents have been added in all wells in the correct order |
| | Too many freeze/thaw cycles or inadequate storage of protein | It is recommended to work with fresh DNMT or nuclear extract samples. If samples need to be stored, small aliquots should be prepared and stored at -80°C to avoid multiple freeze/thaw cycles. During the kit's first use, aliquot the stock His-MBD2b protein into small aliquots and store at -20°C to avoid multiple freeze/thaws |
| | Incorrect assay temperature | Enzymatic reaction should be performed at 37°C. All other incubations can be performed at room temperature. Bring Developing Solution and Stop Solution to room temperature before using |
| | Substrate or conjugate is no longer active | Test conjugate and substrate for activity by mixing a small aliquot of polyHis-HRP and Developing Solution together |
| | Enzyme inhibitor present | Sodium azide will inhibit the peroxidase reaction. Follow our recommendations to prepare buffers |
| | Plate reader settings not optimal | Verify the wavelength and filter settings in the plate reader |
| | Inadequate volume of Developing Solution | Check to make sure that correct volume is delivered by pipette |
| No signal or weak signal in sample wells only | Studying a metal co-activator | The Enzymatic buffer contains EDTA which can interfere with the study of metal co-activators. We recommend preparing a buffer without EDTA as follows: 50 mM Tris base, 1 mM DTT, 5% Glycerol, and 100 µg/ml BSA. The final solution should be pH 7.8. This can be used to perform the enzyme reaction. |
| | Not enough sample per well | Follow the guidelines included in the protocol for the amount of purified DNMT or nuclear extract to use. Titrate your sample to determine the optimal amount to use in the assay. |
| | Protein sample is not properly extracted | Use Active Motif's nuclear extract Kit (Catalog No. 40010) to prepare nuclear extracts. It is recommended to use fresh cells or tissues for extract preparation as frozen samples can lose enzymatic activity. |
| High background in all wells | Developing time too long | Stop enzymatic reaction as soon as the positive wells turn medium-dark blue |
| | Inadequate washing | Ensure all wells are filled with Wash Buffer and follow washing recommendations |
| Uneven color development | Incomplete washing of wells | Ensure all wells are filled with Wash Buffer and follow washing recommendations |
| | Well cross-contamination | Follow washing recommendations |
| High background in sample wells | Too much sample per well | Decrease amount of sample per well. |

## Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.


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