ab156908 – DNA demethylase (total) Activity Quantification Ultra Assay Kit

Instructions for Use

For measuring total DNA demethylase activity/inhibition using nuclear extracts from a broad range of species in a variety of forms including cultured cells and fresh tissues

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

Version 1 Last Updated 1 September 2014

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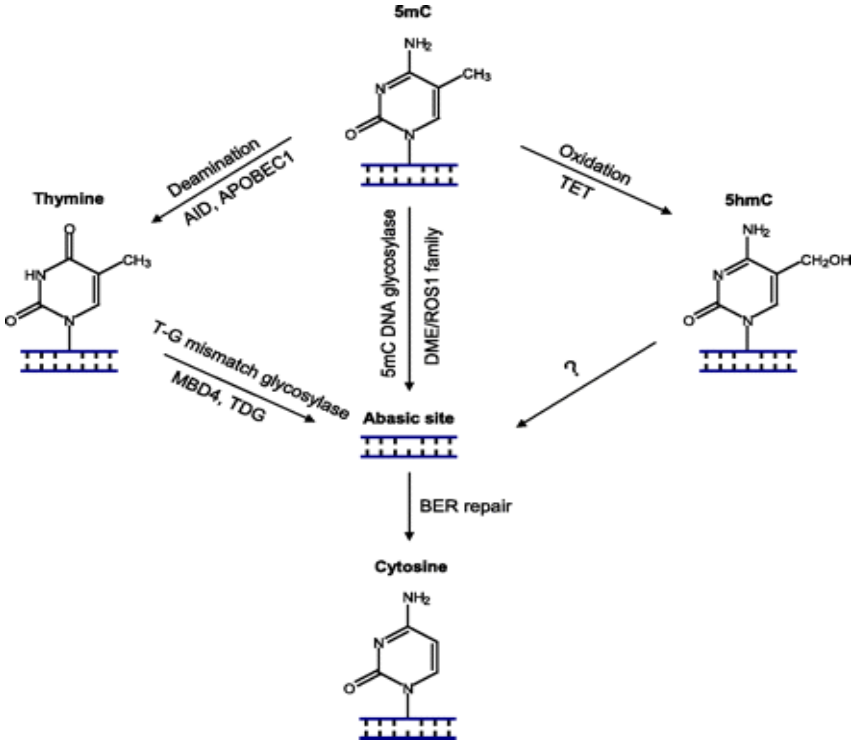
1. BACKGROUND

DNA demethylation is necessary for the epigenentic reprogramming of genes and is also directly involved in many important disease mechanisms such as tumor progression. Demethylation of DNA can either be passive or active, or a combination of both. Passive DNA demethylation usually takes place on newly synthesized DNA strands via DNMT1 during replication rounds. Active DNA demethylation mainly occurs by the removal of 5-methylcytosine through further modified cytosine bases which have been converted by TET enzyme-mediated oxidation. These oxidation products have been shown to be repaired by TDG, a glycosylase which is involved in base excision repair, or directly converted to cytosine by DNMT3A/ DNMT3B in oxidative states. It is proposed that DNA demethylation could also be initiated by deamination of 5-mC through candidate deaminases including AID and APOBEC1, which convert 5-mC to thymine. The resulting thymine could be repaired by BER initiated by a T-G mismatch glycosylase such as MBD4 or TDG. In addition, the 5-mC base can be directly removed in plants by the DME/ROS1 family of 5-mC DNA glycosylases, resulting in an abasic site that is repaired by the BER process.



Currently, there are very few methods available for the detection of DNA demethylase activity/inhibition using nuclear extracts. Abcam's DNA demethylase (total) Activity Quantification Ultra Assay Kit (ab156908) is a further refinement of its predecessors as it enhances sample signals and significantly minimizes background signals, in addition to being five times more sensitive.

This kit has the following advantages:

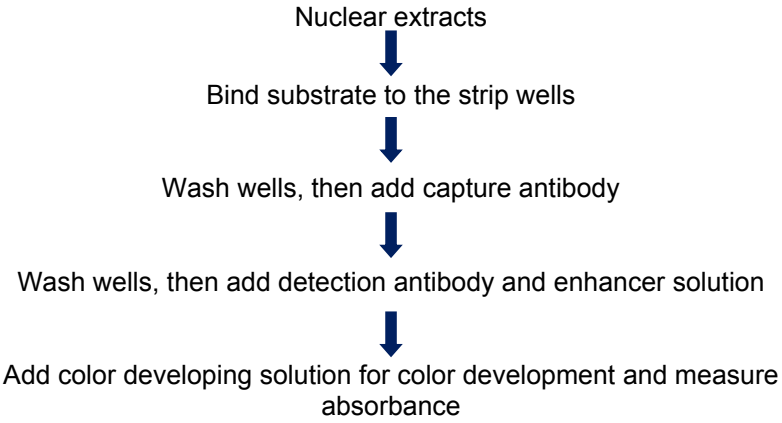
- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire procedure can be completed within 4 hours
- Safe and innovative colorimetric assay without radioactivity, extraction, or chromatography

- An ultra-sensitive detection limit with as low as 1 µg of nuclear extract, which is five times more sensitive than the predecessor kit
- 96 strip-well microplate format allows for either low or high throughput analysis

Abcam's DNA demethylase (total) Activity Quantification Ultra Assay Kit is suitable for measuring total DNA demethylase activity/inhibition using nuclear extracts from a broad range of species such as mammals and plants, in a variety of forms including, but not limited to, cultured cells and fresh tissues.

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 the DNA substrate. The methylated DNA can be recognized with a high affinity 5-methylcytosine antibody and the immuno-signal is enhanced with enhancer solution. 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 and away from light upon receipt.

Observe the storage conditions for individual prepared components in sections 9, 10 & 11.

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

Check if the 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.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	14 mL	28 mL	4°C
Demethylase Assay Buffer	3 mL	6 mL	RT
10X Assay Substrate	10 µL	20 µL	–20°C
Binding Solution	5 mL	10 mL	RT
Assay Control Standard, 20 µg/mL	10 µL	20 µL	–20°C
Capture Antibody,1000 µg/mL	4 µL	8 µL	4°C
Detection Antibody, 400 µg/mL	6 µL	12 µL	–20°C
Enhancer Solution	6 µL	12 µL	–20°C
Developer Solution	5 mL	10 mL	4°C
Stop Solution	5 mL	10 mL	RT
8-Well Assay Strips (With Frame)	6	12	4°C

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
- Microplate reader capable of reading absorbance at 450 nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extract
- Parafilm M or aluminum 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 the volume specified in the table below of 10X Wash Buffer to distilled water and adjust to pH 7.2-7.5.

	Volume to Dilute (mL)	Volume distilled water (mL)	Total Volume (mL)
48 Tests	13	117	130
96 Tests	26	234	260

The 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 1X Assay Substrate

Add 1 µL of 10X Assay Substrate to 9 µL of Demethylase Assay Buffer. About 2 µL of 1X Assay Substrate will be required for each assay well.

9.3 Diluted Capture Antibody

Dilute the Capture Antibody with 1X Wash Buffer at a ratio of 1:1000 (e.g. 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

Dilute the Detection Antibody with 1X Wash Buffer at a ratio of 1:2000 (e.g. add 1 µL of Detection Antibody to 2000 µL of 1X Wash Buffer). About 50 µL of Diluted Detection Antibody will be required for each assay well.

9.5 Diluted Enhancer Solution

Dilute the Enhancer Solution with 1X Wash Buffer at a ratio of 1:5000 (e.g. add 1 µL of Enhancer Solution to 5000 µL of 1X Wash Buffer). About 50 µL of Diluted Enhancer Solution will be required for each assay well.

***Note**: Keep each of 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.*

Approximate amount of required buffers and solutions for defined assay wells based on the protocol:

Reagents	1 Well	1 Strip (8 Wells)	2 Strips (16 Wells)	6 Strips (48 Wells)	12 Strips (96 Wells)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL	240 mL
Demethylase Assay Buffer	50 µL	400 µL	800 µL	2400 µL	4800 µL
1X Assay Substrate	2 µL	16 µL	32 µL	96 µL	192 µL
Binding Solution	80 µL	650 µL	1350 µL	2700 µL	5400 µL
Assay Control Standard, 5 µg/µL	N/A	N/A	4 µL (optional)	8 µL	8 µL
Diluted Capture Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Diluted Detection Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Diluted Enhancer Solution	50 µL	400 µL	800 µL	2400 µL	4800 µL
Developer Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL	9.6 mL
Stop Solution	0.1 mL	0.8 mL	1.6 mL	4.8 mL	9.6 mL

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be 2 µg to 20 µg with an optimal range of 5-10 µg.

Nuclear Extraction: You can use your method of choice for preparing nuclear extracts. Abcam offers a Nuclear Extraction Kit (ab113474) optimized for use with this kit. Nuclear extracts should be stored in aliquots at -80°C until use.

11. STANDARD PREPARATION

Suggested Standard Curve Preparation:

11.1 Dilute the Assay Control Standard with Demethylase Assay Buffer to 5 ng/µL by adding 1 µL of the Assay Control Standard to 4 µL of Demethylase Assay Buffer.

11.2 Then, further prepare five concentrations by combining the 5 ng/µL Diluted Assay Control Standard with Demethylase Assay Buffer into final concentrations of 0.2, 0.5, 1.0, 2.0, and 50 ng/µL according to the following dilution chart:

Tube	Assay Control Standard (5 ng/µL) (µL)	Demethylase Assay Buffer (µL)	Resulting Assay Control Standard Concentration (ng/µL)
1	1.0	24.0	0.2
2	1.0	9.0	0.5
3	1.0	4.0	1.0
4	2.0	3.0	2.0
5	4.0	0.0	5.0

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.*

12. PLATE PREPARATION

The suggested strip-well plate setup for standard curve preparation in a 48-assay format (in a 96-assay format, Strips 7 to 12 can be configured as Sample). The controls and samples can measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	Assay Standard Control 0.2 ng/µL	Assay Standard Control 0.2 ng/µL	Sample	Sample	Sample	Sample
C	Assay Standard Control 0.5 ng/µL	Assay Standard Control 0.5 ng/µL	Sample	Sample	Sample	Sample
D	Assay Standard Control 1 ng/µL	Assay Standard Control 1 ng/µL	Sample	Sample	Sample	Sample
E	Assay Standard Control 2 ng/µL	Assay Standard Control 2 ng/µL	Sample	Sample	Sample	Sample
F	Assay Standard Control 5 ng/µL	Assay Standard Control 5 ng/µL	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

13. ASSAY PROCEDURE

- **Internal Control: An assay standard is provided in this kit for the quantification of DNA demethylase activity. Because DNA demethylase 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**

13.1 Enzymatic Reaction

13.1.1 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).

13.1.2 Add 80 µL of Binding Solution to each well.

13.1.3 Add 2 µL of 1X Assay Substrate into each sample well. Add 2 µL of Demethylase Assay Buffer into blank wells. Add 1 µL of Diluted Assay Control Standard into the standard curve wells (see the designated wells depicted Section 12 – Plate Preparation). Mix solution by gently tilting from side to side or shaking the plate several times. Ensure the solution coats the bottom of the well evenly.

Note: *For the standard curve, add 1 µL of Diluted Assay Control Standard at concentrations of 0.2-5 ng/µL (see Section 11 - Standard Preparation). The final concentrations should be 0.2, 0.5, 1, 2 and 5 ng per well.*

13.1.4 Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 90 minutes.

13.1.5 Remove the reaction solution from each well.

13.1.6 Wash each well three times with 150 µL of 1X Wash Buffer each time.

13.1.7 Blank Wells: Add 50 µL of Demethylase Assay Buffer to each blank well.

13.1.8 Standard Wells: Add 50 µL of Demethylase Assay Buffer to each standard well.

13.1.9 Control Sample Wells without Nuclear Extracts: Add 45-48 µL of Demethylase Assay Buffer and 2-5 µL of your protein extraction buffer.

13.1.10 Sample Wells Without Inhibitor: Add 46-49 µL of Demethylase Assay Buffer and 1-4 µL of nuclear extracts to each sample well without inhibitor. Total volume should be 50 µL per well.

13.1.11 Sample Wells With Inhibitor: Add 41-44 µL of Demethylase Assay Buffer, 1-4 µL of nuclear extracts and 5 µL of inhibitor solution. Total volume should be 50 µL per well.

Note: *(1) Follow the suggested well setup diagrams (Section 12 – Plate Preparation);(2) It is recommended to use 5 µg to 10 µg of nuclear extract per well;(3) The concentration of inhibitor to be added into the sample wells can be varied (1 µM to 1000 µM). However, the final concentration of the inhibitors before adding to the wells should be prepared with Demethylase Assay Buffer at a 1:10 ratio (e.g., add 0.5 µL of inhibitor to 4.5 µL of Demethylase Assay Buffer) so that the original solvent of the inhibitor can be reduced to 1% of the reaction solution or less*

13.1.12 Tightly cover strip plate with Parafilm M to avoid evaporation and incubate at 37°C for 60-90 minutes.

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

13.2 **Antibody Binding and Signal Enhancing**

13.2.1 Add 50 µL of the Diluted Capture Antibody to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 60 minutes.

13.2.2 Remove the Diluted Capture Antibody solution from each well.

13.2.3 Wash each well three times with 150 µL of 1X Wash Buffer each time.

13.2.4 Add 50 µL of the Diluted Detection Antibody to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.5 Remove the Diluted Detection Antibody solution from each well.

13.2.6 Wash each well four times with 150 µL of 1X Wash Buffer each time.

13.2.7 Add 50 µL of the Diluted Enhancer Solution to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 minutes.

13.2.8 Remove the Diluted Enhancer Solution from each well.

13.2.9 Wash each well five times with 150 µL of 1X Wash Buffer each time.

Note: *Ensure any residual wash buffer in the wells is thoroughly removed at each wash step. The wash can be carried out by simply pipetting the wash buffer into the wells and then pipetting the buffer out from the wells (discard the buffer).*

13.3 **Signal Detection**

13.3.1 Add 100 µL of the Developer Solution to each well and incubate at room temperature for 1-10 minutes away from light. Begin monitoring color changes in the sample wells and control wells. The Developer Solution will turn blue in the presence of sufficient methylated DNA.

13.3.2 Add 100 µL of Stop Solution to each well to stop enzyme reaction when the color in the positive control wells turns medium blue. The color will change to yellow after adding the Stop Solution and the absorbance should be read on a microplate reader within 2-10 minutes at 450 nm with an optional reference wavelength of 655 nm.

Note: *(1) Most microplate readers have capability to carry out dual wavelength analysis and will automatically subtract 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 manually 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.*

13.3.3 Calculate DNA demethylase activity or inhibition using the formulae provided in Section 13 – Data Analysis.

14. ANALYSIS

Calculate average duplicate readings for sample wells and blank wells. Calculate the total DNA demethylase activity or inhibition using the following formula:

Demethylase activity (OD/h/mg) =

$$\frac{\text{OD (Control* – Blank) – OD (Sample – Blank)}}{\text{(Protein amount (µg)**/1000) x Incubation time***}}$$

*Control: sample wells without nuclear extracts.
*Protein amount (µg) added into the reaction at step 13.1.10.
**Incubation time (hours) at step 13.1.12.

Example calculation:

Average OD450 of control is 0.55.
Average OD450 of sample is 0.25.
Average OD450 of blank is 0.05.
Protein amount is 5 µg
Incubation time is 1 hours

DNA demethylase activity = [(0.5– 0.2) / (5/1000 x 1)]
= 60 OD/h/mg

For an accurate or specific activity calculation, generate a standard curve and plot OD versus the amount of Assay Control Standard at each concentration point. Determine the slope as delta OD/ng then calculate the DNA demethylase activity or inhibition using the following formulae:

Activity (ng/h/mg) =

$$\frac{(\text{Control* OD} - \text{Blank OD}) - (\text{Sample OD} - \text{Blank OD})}{\text{Slope} \times \text{Protein amount (µg)**} \times \text{Incubation time***}} \times 1000$$

*Control: sample wells without nuclear extracts.
*Protein amount (µg) added into the reaction at step 13.1.10.
**Incubation time (hours) at step 13.1.12.

For inhibition calculation:

Inhibition % =

$$\frac{(\text{Control OD} - \text{Blank OD}) - (\text{Inhibitor OD} - \text{Blank OD})}{(\text{Control OD} - \text{Blank OD}) - (\text{No Inhibitor OD} - \text{Blank OD})} \times 100\%$$

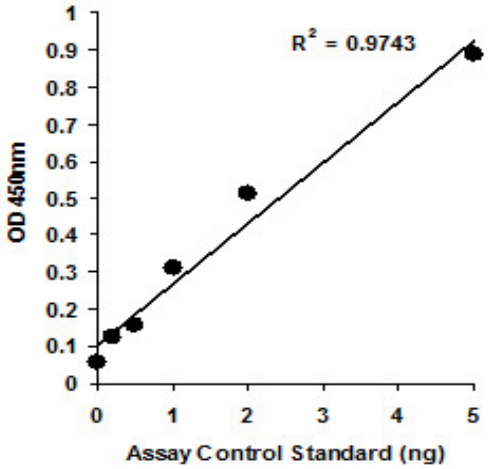


Figure 1. Assay control standard was added into the assay wells at different concentrations and then measured with Abcam's DNA demethylase (total) Activity Quantification Ultra Assay Kit ab156908.

15. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in both the standard 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 substrate and standard are not properly bound to the wells	Ensure that (1) the Demethylase Assay Buffer and Assay Control Standard are added into the wells; (2) the wells are completely covered with sufficient Binding Solution; and (3) binding time is sufficient (90 minutes)
	Incubation time and temperature are incorrect	Ensure the incubation time and temperature described in the protocol are followed correctly
	Incorrect absorbance reading	Check if the 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 temperatures and the cap is tightly capped after each opening or use
No signal or weak signal in only the standard curve wells	The standard amount is insufficiently added to the well in step 13.1.3	Ensure a sufficient amount of standard is added

	The standard is degraded due to improper storage conditions	Follow the Storage and Stability guidance of this User Guide for storage of the Assay Control Standard
High background present in the blank wells	Insufficient washing of wells	Check if washing at each step is performed according to the protocol
	Contaminated by sample or standard	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips
	Incubation time with detection antibody is too long	The incubation time at step 13.2.4 should not exceed 45 minutes
	Over development of color	Decrease the development time in step 13.3.1 before adding Stop Solution in step 13.3.2
No signal or weak signal only in sample wells	Sample is not properly extracted or purified	Ensure your protocol is suitable for DNA demethylase extraction. For the best results, it is advised to use Abcam's Nuclear Extraction Kit (ab113474). 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 nuclear extracts is used as indicated in steps 13.1.10 and 13.1.11. 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 -80°C, with no more than 6 weeks for nuclear extracts. Avoid repeated freezing/thawing
	Little or no activity of DNA demethylase 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
Uneven color development	Insufficient washing of the wells	Ensure the wells are washed according to the user guide. Ensure residual wash buffer is removed as much as possible
	Delayed color development or delayed stopping of color development in the wells	Ensure color development and stop solutions are added sequentially and consistent with the order you added the other reagents (e.g., from well A to G or from well 1 to 12)

16. NOTES

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