ab113470 – DNMT3A Assay Kit

Instructions for Use

For the measurement of DNMT3A amounts from human and mouse tissues or cells

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

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1. BACKGROUND

Epigenetic inactivation of genes plays a critical role in many important human diseases, especially in cancer. A core mechanism for epigenetic inactivation of the genes is methylation of CpG islands in genome DNA. Methylation of CpG islands involves the course in which DNA methyltransferases (DNMTs) transfer a methyl group from S-adenosyl-L-methionine to the fifth carbon position of the cytosines. At least three families of DNMTs have been so far identified in mammals: DNMT1, DNMT2, and DNMT3. The DNMT3 family comprises of three different proteins: DNMT3A, DNMT3B, and DNMT3L. DNMT3A and DNMT3B have been demonstrated to methylate both unmethylated and hemimethylated DNA equally and are supposed to mediate de novo methylation together with DNMT1. Increased activation or amounts of DNMT3 is believed to be involved in carcinogenesis, and other genetic and epigenetic diseases.

Several methods, such as Western blot, are used for measuring levels of DNMT3A. However, these methods available so far are inconvenient, considerably time consuming, labor intensive, or have low throughput. ab113470 addresses these problems by using a unique procedure to measure the amount of DNMT3A.

The kit has the following features:

- Extremely fast procedure, which can be finished within 3.5 hours
- Innovative colorimetric assay, which quantitatively measures the amount of DNMT3A without the need for electrophoresis
- Strip microplate format makes the assay flexible, allowing manual or high throughput analysis
- Simple, reliable, and consistent assay conditions

The DNMT3A Assay Kit is designed for measuring total DNMT3A amount from human/mouse tissues or cells. In an assay with this kit, the unique DNMT affinity substrate is stably coated on the strip well. The sample is added into the well and DNMT3A contained in the sample binds to the substrate. The bound DNMT3A can be recognized with a specific DNMT3A

antibody and is colorimetrically quantified through an ELISA-like reaction. The amount of DNMT3A is proportional to the intensity of color development.

2. ASSAY SUMMARY

Prepare nuclear extract

↓

Incubate with capture reagent and assay buffer

↓

Add affinity antibody after washing wells

↓

Add detection antibody after washing wells

↓

Add developing solution for color development, then measure absorbance

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. The kit is stable for up to 6 months from the shipment date, when stored properly.

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.

Check if a blue color is present in Developing Solution, which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of Developing Solution required into a secondary container (tube or vial) before adding Developing Solution into the assay wells.

5. MATERIALS SUPPLIED

Item	48 Tests	96 Tests	Storage Condition (Before Preparation)
10X Wash Buffer	12 mL	25 mL	4°C
Assay Buffer	5 mL	10 mL	RT
DNMT3A Standard, 20 µg/mL*	16 µL	30 µL	-20°C
Affinity Antibody, 100 µg/mL*	8µL	16 µL	4°C
Detection Antibody, 200 µg/mL*	10 µL	20 µL	-20°C
Developing Solution	6 mL	12 mL	4°C
Stop Solution	6 mL	11 mL	RT
Blocking Buffer)	10 mL	20 mL	4°C
8-Well Assay Strips (with Frame)	6	12	4°C

*After thawing, 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
- Microplate reader capable of reading absorbance at 450 nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Distilled water
- Nuclear extracts
- 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 the volume specified in the table below of 10X Wash Buffer to distilled water (pH to 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 Affinity Antibody Solution

Dilute Affinity Antibody with 1X Wash Buffer at a ratio of 1:500 (i.e., add 1 µL of Affinity Antibody to 500 µL of 1X Wash Buffer). 50 µL of Affinity Antibody Solution will be required for each assay well.

9.3 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). 50 µL of Detection Antibody Solution will be required for each assay well.

9.4 DNMT3A Standard

Suggested Standard Curve Preparation: Dilute 20 ng/µL DNMT3B Standard with Assay Buffer to the concentrations of 1, 2, 5, 10 and 20 ng/µL according to the table below.

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.*

Table 1. Suggested Standard Curve Preparation

Tube	DNMT3A Standard (µL)	Assay Buffer (µL)	Concentration of DMNT3A (ng/µL)
1	0.5	9.5	1
2	0.5	4.5	2
3	1.0	3.0	5
4	2.0	2.0	10
5	4.0	0.0	20

Table 2. 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)	96 wells (12 strips)
1X Wash Buffer	2.5 mL	20 mL	40 mL	120 mL	240mL
Assay Buffer	100 µL	800 µL	1600 µL	4900 µL	9600 µL
Blocking Buffer	0.15 mL	1.2 mL	2.5 mL	7.5 mL	14.5 mL
DNMT3A Standard	N/A	N/A	4 µL	8 µL	8 µL
Affinity Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Detection Antibody	50 µL	400 µL	800 µL	2400 µL	4800 µL
Developing Solution	100 µL	800 µL	1600 µL	4800 µL	9600 µL
Stop Solution	100 µL	800 µL	1600 µL	4800 µL	9600 µL

10. SAMPLE PREPARATION

Input Amount: The amount of nuclear extracts for each assay can be between 1 µg and 20 ug with an optimal range of 5 to 10 µg.

Nuclear Extraction: You can use your method of choice for preparing nuclear extracts from the treated and untreated samples. For your convenience and the best results, Abcam offers the Nuclear Extraction Kit (ab113474) optimized for use with this kit. Nuclear extracts can be used immediately or stored at –80°C for future use.

11. PLATE PREPARATION

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

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Blank	Blank	Sample	Sample	Sample	Sample
B	DNMT3A Standard 2 ng	DNMT3A Standard 2 ng	Sample	Sample	Sample	Sample
C	DNMT3A Standard 4 ng	DNMT3A Standard 4 ng	Sample	Sample	Sample	Sample
D	DNMT3A Standard 10 ng	DNMT3A Standard 10 ng	Sample	Sample	Sample	Sample
E	DNMT3A Standard 20 ng	DNMT3A Standard 20 ng	Sample	Sample	Sample	Sample
F	DNMT3A Standard 40 ng	DNMT3A Standard 40 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

12. ASSAY PROCEDURE

12.1 Pre-determine the number of strip wells required for your experiment. It is advised to run replicate samples (include blank and positive controls) 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).

12.2 Blank Wells: Add 100 µLof Assay Buffer to each blank well.
Standard Wells: Add 98 µL of Assay Buffer and 2 µL of Diluted DNMT3A Standard to each standard well with a minimum of five wells, each at a different concentration between 2 and 40 ng/µL See Table 2
Sample Wells: Add 94 to 98 µL of Assay Buffer and 2 to 6 µL of your nuclear extracts to each sample well. Total volume should be 100 µL per well.

Notes: *Follow the suggested well setup diagrams is recommended to use 5 µg to 10 µg of nuclear extract per well.*

12.3 Cover strip-well microplate with Parafilm M or aluminum foil to avoid evaporation and incubate at 37°C for 90 to 120 min.

12.4 Remove the reaction solution from each well. Add 150 µL of Blocking Buffer to each well, then cover with Parafilm M or aluminum foil and incubate at 37°C for 30 min.

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

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

12.7 Remove the Diluted Affinity Antibody solution from each well.

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

12.9 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 min.

12.10 Remove the Diluted Detection Antibody solution from each well.

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

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

12.12 Add 100 µL of Developing Solution to each well and incubate at room temperature for 1 to 10 min away from light. Begin monitoring color change in the sample wells and control wells. The Developing Solution will turn blue in the presence of sufficient demethylated products.

12.13 Add 100 µL of Stop Solution to each well to stop enzyme reaction when color in the positive control wells turns medium blue. The color will change to yellow after adding Stop Solution and the 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 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 strip-well 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 DNMT3A change using the following formula:

$$\text{DMNT3A change \%} = \frac{\text{Treated (Tested) Sample OD} - \text{Blank OD}}{\text{Untreated (Control) Sample OD} - \text{Blank OD}} \times 100\%$$

Example calculation:

Average OD450 of treated sample is 0.5

Average OD450 of untreated sample is 0.9

Average OD450 of blank is 0.1

DNMT3A change % = [(0.5 – 0.1)/ (0.9 – 0.1)] x 100%

= 50 %

For detailed quantification:

Generate a standard curve and plot OD value versus amount of DNMT3A Standard at each concentration point.

Determine the slope as OD/ng (you can use Microsoft Excel statistical functions for slope calculation), then calculate the amount of DNMT3B using the following formulas:

$$\text{DNMT3B (ng/mg protein)} = \frac{\text{(Sample OD – Blank OD)}}{\text{Slope x Protein Amount (µg*)}} \text{ X } 1000$$

*Nuclear extract added into sample wells at step 12.2.

14. TROUBLESHOOTING

Problem	Cause	Solution
No Signal for Both the Positive Control and the Samples	Reagents are added incorrectly	Check if reagents are added in order and if any steps of the procedure may have been omitted by mistake
	Incubation time and temperature are incorrect	Ensure the incubation time and temperature described in the protocol are followed correctly
No Signal or Very Weak Signal for only the positive control	The standard protein is insufficiently added to the well	Ensure a sufficient amount of standard protein is added
	The DNMT3A standard has lost the binding activity due to incorrect storage	Follow the guidance in the protocol for storage of DNMT3A Standard
No Signal for Only the Sample	The protein amount is added into well insufficiently	Ensure extract contains a sufficient amount of protein
	Nuclear extracts are incorrectly stored	Ensure the nuclear extracts are stored at –80°C
High Background Present for the Blank	The well is not washed sufficiently	Check if wash at each step is performed according to the protocol

High Background Present for the Blank	Contaminated by the DNMT3A standard	Ensure the well is not contaminated from adding DNMT3A standard accidentally or from using DNMT3A contaminated tips
	Overdevelopment	Decrease development time in step 12.12
Uneven color development	Insufficient washing of the wells.	Ensure the wells are washed according to the guidance of washing and residue washing buffer is removed as much as possible
	Delayed color development or delayed stopping of color development in the wells	Ensure color development solution or stop solution is added sequentially and is 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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