

EpiQuik™ DNMT3A Assay Kit

Base Catalog # P-3012

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

Uses: The EpiQuik™ DNMT3A Assay Kits are very suitable for measuring Dnmt3A amounts quantitatively from fresh tissue and cultured cells of human and mouse.

 **EPIGENETEK**
Real Solutions for Epigenetic Research

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Page 1

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P-3012

KIT CONTENTS

Component	48 Assays Cat. #P-3012-2	96 Assays Cat. #P-3012-3	Storage Upon Receipt
DA1 (10X Wash Buffer)	14 ml	28 ml	4°C
DA2 (Assay Buffer)	5 ml	10 ml	4°C
DA3 (DNMT3A Standard, 20 µg/ml)*	16 µl	30 µl	-20°C
DA4 (Capture Antibody, 100 µg/ml)*	8 µl	16 µl	4°C
DA5 (Detection Antibody, 200 µg/ml)*	10 µl	20 µl	-20°C
DA6 (Developing Solution)	6 ml	12 ml	4°C
DA7 (Stop Solution)	6 ml	11 ml	RT
BB (Blocking Buffer)	10 ml	20 ml	4°C
8-Well Assay Strips (With Frame)	6	12	4°C
User Guide	1	1	RT

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

SHIPPING & STORAGE

The kit is shipped in two parts: one part at ambient room temperature, and the second part on frozen ice packs at 4°C.

Upon receipt: (1) Store **DA3** and **DA5** at -20°C away from light; (2) Store **DA1**, **DA2**, **DA4**, **DA6**, **Blocking buffer** and the **8-Well Assay Strips** at 4°C away from light; (3) Store **all other components** at room temperature. The kit is stable for up to 6 months from the shipment date, when stored properly.

Note: (1) Check if wash buffer, **DA1**, contains salt precipitates before using. If so, warm (at room temperature or 37°C) and shake the buffer until the salts are re-dissolved; (2) check if a blue color is present in **DA6** (Developing Solution), which would indicate contamination of the solution and should not be used. To avoid contamination, transfer the amount of **DA6** required into a secondary container (tube or vial) before adding **DA6** into the assay wells.

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 extracts
- Parafilm M or aluminum foil

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of the EpiQuik™ DNMT3A Assay Kit is tested against predetermined specifications to ensure consistent product quality. Epigenetek 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: Epigenetek 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 EpiQuik™ DNMT3A Assay Kit is for research use only and is not intended for diagnostic or therapeutic applications.

Intellectual Property: The EpiQuik™ DNMT3A Assay Kit and methods of use contain proprietary technologies by Epigenetek.

A BRIEF OVERVIEW

Epigenetic inactivation of genes play 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: DNMT 3A, DNMT3B, and DNMT3L. DNMT3A and DNMT3B have been demonstrated to methylate both unmethylated and hemimethylated DNA equally and is 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.

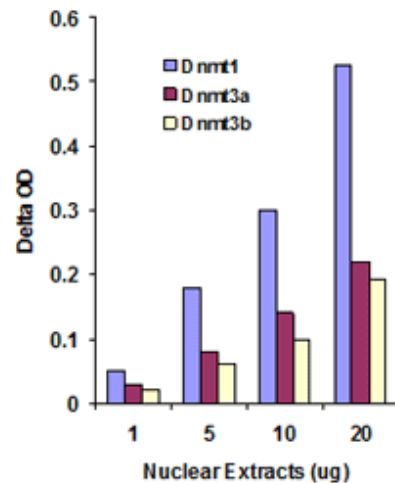
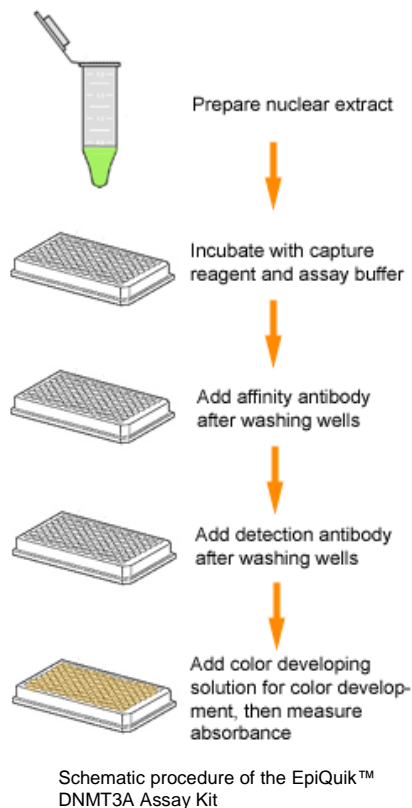
Several methods, such as Western blot, are used for measuring levels of Dnmt3A. However, these methods available so far are inconvenient, considerably time consuming, labor intensive, or have low throughput. The EpiQuik™ Dnmt3A Assay Kit addresses these problems by using a unique procedure to measure the amount of Dnmt3A. The kit has the following features:

- Very rapid procedure, which can be finished within 3.5 hours.
- Innovative colorimetric assay to quantitatively measure the amount of DNMT3A without the need for electrophoresis.
- Strip microplate format makes the assay flexible: manual or high throughput analysis.

- Simple, reliable, and consistent assay conditions.

PRINCIPLE & PROCEDURE

The EpiQuik™ DNMT3A Assay Kit is designed for measuring total DNMT3A amount from 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 colorimetrically quantified through an ELISA-like reaction. The amount of DNMT3A is proportional to the intensity of color development.



Nuclear extracts were prepared from MCF-7 cells using the EpiQuik Nuclear Extraction Kit and total amount of DNMT was measured.

PROTOCOL

Starting Materials

Input Amount: The amount of nuclear extracts for each assay can be between 1 µg and 20 µg 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. Epigentek also offers a nuclear extraction kit (Cat # OP-0002) optimized for use with this kit.

Nuclear extracts should be stored at -80°C in aliquots until use.

1. Working Buffer & Solution Preparation

- a. Prepare **Diluted DA1** 1X Wash Buffer:

48-Assay Kit: Add 13 ml of **DA1** 10X Wash Buffer to 117 ml of distilled water and adjust pH to 7.2-7.5.

96-Assay Kit: Add 26 ml of **DA1** 10X Wash Buffer to 234 ml of distilled water and adjust pH to 7.2-7.5.

This **Diluted DA1** 1X Wash Buffer can now be stored at 4°C for up to six months.

- b. Prepare **Diluted DA4** Capture Antibody Solution:

Dilute **DA4** Capture Antibody with **Diluted DA1** 1X Wash Buffer at a ratio of 1:500 (i.e., add 1 μl of **DA4** to 500 μl of **Diluted DA1**). 50 μl of **Diluted DA4** will be required for each assay well.


- c. Prepare **Diluted DA5** Detection Antibody Solution:

Dilute **DA5** Detection Antibody with **Diluted DA1** 1X Wash Buffer at a ratio of 1:2000 (i.e., add 1 μl of **DA5** to 2000 μl of **Diluted DA1**). 50 μl of **Diluted DA5** will be required for each assay well.

- e. Prepare **Diluted DNMT3A Standard**:

Suggested Standard Curve Preparation: Dilute **DA3** DNMT3A Standard with **DA2** to the concentrations of 1, 2, 5, 10 and 20 $\text{ng}/\mu\text{l}$ according to the following dilution chart:

Tube	DA3 (20 $\text{ng}/\mu\text{l}$)	DA2	Resulting DA3 Concentration
1	0.5 μl	9.5 μl	1 $\text{ng}/\mu\text{l}$
2	0.5 μl	4.5 μl	2 $\text{ng}/\mu\text{l}$
3	1.0 μl	3.0 μl	5 $\text{ng}/\mu\text{l}$
4	2.0 μl	2.0 μl	10 $\text{ng}/\mu\text{l}$
5	4.0 μl	0.0 μl	20 $\text{ng}/\mu\text{l}$

 *Note: Keep each of the diluted solutions except **DA1** 1X Wash Buffer on ice until use. Any remaining diluted solutions other than **Diluted DA1** should be discarded if not used within the same day.*

2. DNMT3A Binding

- a. Predetermine 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).

- b. Blank Wells: Add 100 μl of **DA2** to each blank well.

- c. **Standard Wells:** Add 98 μ l of **DA2** and 2 μ l of **Diluted DNMT3A Standard** to each standard well with a minimum of five wells, each at a different concentration between 2 and 40 ng/ μ l (based on the dilution chart in Step 1e; see Table 2 on Page 8 as an example).
- d. **Sample Wells:** Add 94 to 98 μ l of **DA2** and 2 to 6 μ l of your nuclear extracts to each sample well. Total volume should be 100 μ l per well.

Note: (1) Follow the suggested well setup diagrams on page 8; (2) It is recommended to use 5 μ g to 10 μ g of nuclear extract per well.

- e. Cover strip-well microplate with Parafilm M or aluminum foil to avoid evaporation and incubate at 37°C for 90 to 120 min.
- f. Remove the reaction solution from each well. Add 150 μ l of **BB** Blocking Buffer to each well, then cover with Parafilm M or aluminum foil and incubate at 37°C for 30 min.
- g. Remove the reaction solution from each well. Wash each well three times with 150 μ l of **the Diluted DA1 1X Wash Buffer** each time

3. Antibody Binding & Signal Enhancing

- a. Add 50 μ l of the **Diluted DA4** to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 60 min.
- b. Remove the **Diluted DA4** solution from each well.
- c. Wash each well three times with 150 μ l of the **Diluted DA1** each time.
- d. Add 50 μ l of the **Diluted DA5** to each well, then cover with Parafilm M or aluminum foil and incubate at room temperature for 30 min.
- e. Remove the **Diluted DA5** solution from each well.
- f. Wash each well four times with 150 μ l of the **Diluted DA1** each time.

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

4. Signal Detection

- a. Add 100 μ l of **DA6** 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 **DA6** solution will turn blue in the presence of sufficient demethylated products.
- b. Add 100 μ l of **DA7** to each well to stop enzyme reaction when 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 **DA7** 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.

5. DNMT3A Calculation

- Calculate the average duplicate readings for the sample wells and blank wells.
- Calculate % DNMT3A change using the following formula:

$$\text{DNMT3A change \%} = \frac{\text{Treated (Tested) Sample OD} - \text{Blank OD}}{\text{Untreated (Control) Sample OD} - \text{Blank OD}} \times 100\%$$

Example calculation:

Average OD450 of treated sample is 0.5
 Average OD450 of untreated control is 0.9
 Average OD450 of blank is 0.1

$$\text{DNMT3A change \%} = \frac{(0.5 - 0.1)}{0.9 - 0.1} \times 100\% = 50\%$$

For Detailed Quantification :

- Generate a standard curve and plot OD value versus amount of **DA3 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 DNMT3A using the following formulas:

$$\text{DNMT3A (ng/mg protein)} = \frac{(\text{Sample OD} - \text{Blank OD})}{\text{Slope} \times \text{Protein Amount (ug*)}} \times 1000$$

* Nuclear extract added into sample wells at Step 2d.

SUGGESTED BUFFER AND SOLUTION SETUP

Table 1. 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)
Diluted DA1	2.5 ml	20 ml	40 ml	120 ml	240 ml
DA2	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
DA3 Standard control	N/A	N/A	4 µL (optional)	8 µl	8 µl
Diluted DA4	50 µl	400 µl	800 µl	2400 µl	4800 µl

Diluted DA5	50 µl	400 µl	800 µl	2400 µl	4800 µl
DA6	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
DA7	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml

SUGGESTED STRIP WELL SETUP

Table 2. The suggested strip-well plate setup for Dnmt1 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	DA3 2 ng	DA3 2 ng	Sample	Sample	Sample	Sample
C	DA3 4ng	DA3 4ng	Sample	Sample	Sample	Sample
D	DA3 10 ng	DA3 10 ng	Sample	Sample	Sample	Sample
E	DA3 20 ng	DA3 20 ng	Sample	Sample	Sample	Sample
F	DA3 40 ng	DA3 40 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

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.
	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) 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 standard curve wells	The standard amount is insufficiently added to the well in Step 2c.	Ensure a sufficient amount of standard is added.
	The standard is degraded due to improper storage conditions.	Follow the Shipping & Storage guidance in this User Guide for storage of DA3 DNMT3A Standard .
High background present in the blank	Insufficient washing of wells.	Check if washing recommendations at each step is performed according

wells		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 Diluted DA5 is too long.	The incubation time at Step 3d should not exceed 90 min.
	Over-development of color.	Decrease the development time in Step 4a before adding DA6 Stop Solution in Step 4b.
No signal or weak signal only in sample wells	Protein sample is not properly extracted or purified.	Ensure your protocol is suitable for histone protein extraction. For the best results, it is advised to use Epigentek's Nuclear extraction Kit (Cat. No. OP-0002).
	Sample amount added into the wells is insufficient.	Ensure a sufficient amount of nuclear extracts is used as indicated in Step 2. 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 months for nuclear extracts.
	Little or no DNMT3A 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 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).
Large variation between replicate wells	Color reaction is not evenly stopped due to an inconsistency in pipetting time.	Ensure DA6 Developer Solution and DA7 Stop Solution are added at the same time between replicates or otherwise maintain consistent timing between each addition of solutions.
	Color reaction is not evenly stopped due to an inconsistent order when adding solutions.	Ensure all solutions, particularly DA6 Developer Solution and DA7 Stop Solution, are added in the same order

		each time as all other solutions.
	The solutions are not evenly added due to an 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 (ex: 1 ul) are completely added into the wells.
	Solutions or antibodies not actually added into the wells.	Do not allow pipette tip to touch the outer edges or inner sides of the wells in order to prevent solutions from sticking to the surface.
	Did not sufficiently shake the solutions in the wells after adding DA7 Stop Solution in Step 4b.	Gently and evenly shake the plate frame across a flat surface so that the solutions in the wells are better distributed. Do not stir.
	Did not use the same 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.

RELATED PRODUCTS

Nuclear Extract Preparation

OP-0002 EpiQuik™ Nuclear Extraction Kit

DNA Methyltransferase Activity/Inhibition Assay

P-3009 EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Colorimetric)

P-3010 EpiQuik™ DNMT Activity/Inhibition Assay Ultra Kit (Fluorometric)

P-3011 EpiQuik™ DNMT1 Assay Kit

P-3012 EpiQuik™ DNMT3B Assay Kit

