ab156904 – 5-methylcytosine Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of total urinary 5-methylcytosine levels using fresh or frozen urine from humans and animals

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

Version 1 Last Updated 3 September 2014

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1. BACKGROUND

Nucleobase 5-methylcytosine (5-mC), a modified form of cytosine converted by cytosine methyltransferases is widespread both in DNA and different cellular RNAs. The biological importance of DNA 5-mC-methylation as a major epigenetic modification in phenotype and gene expression has been recognized widely. Recent data strongly suggests that RNA 5-mC methylation is also involved in the regulation of various biological processes including tRNA stability/recognition and mRNA translation.

Urinary excretion of 5-mC including both 5-methyl-2-deoxycytidine and 5-methylcytidine is an indication of the whole body turnover or degradation of methylated DNA and RNA. The urinary 5-mC level can be changed with a change of the bodies' turnover of methylated DNA/RNA or alteration of cellular DNA/RNA methylation status. A number of studies have indicated that 5-mC excreted in urine has the potential to act as a cancer biomarker, with an increased level arising from disease onset and progression. For example, an elevated level of urinary 5-mC was observed in lung cancer, breast cancer, and leukemia patients with active disease states. It was also shown that urinary 5-mC excretion is effected by Alzheimer's disease or by radiation treatment. It has been suggested that urinary 5-mC might be applicable as a biological marker for detecting certain types of cancer and monitoring cancer progression after treatment with radiation or demethylation reagents.

Chromatography-based techniques such as HPLC and TLC mass spectrometry are used for detecting 5-mC in urine. These methods are accurate but are time consuming, less sensitive, and have low throughput with high costs. To address this problem, Abcam offers the Urine 5-methylcytosine Quantification Kit (Fluorometric) (ab156904) to quantify 5-mC or body turnover status of methylated DNA/RNA using urine samples.

The kit has the following advantages and features:

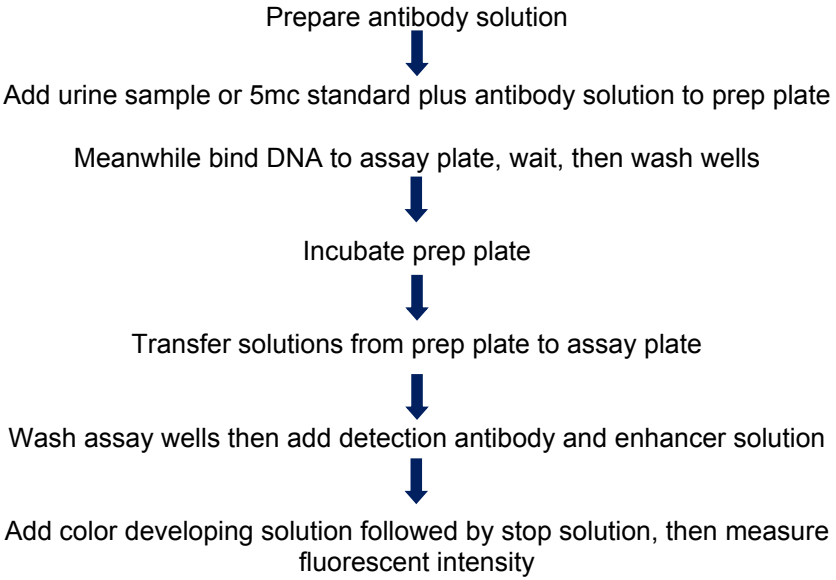
- Innovative fluorometric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 4 hours
- 96 strip well microplate format makes the assay flexible: manual or high throughput analysis
- Innovative kit composition enables background signals to be extremely low, which eliminates the need for plate blocking and allows the assay to be simple, accurate, reliable, and consistent
- The level of 5-mC measured in human urine samples using this kit is comparable to that detected by HPLC method
- A novel assay principle allows high sensitivity to be achieved. The detection limit can be as low as 0.01 ng/assay well or 0.3 nM of 5-mC
- Low input range of urine for each assay with a volume of 0.5-5 µL and an optimal volume of 1 µL
- Optimized antibody and enhancer solutions allow high specificity to 5-mC, with no cross-reactivity to unmethylated cytosine
- Negative control and positive standard are included, which are suitable for quantification of 5-mC in free form and 5-mC contained in methylated DNA/RNA fragments from different urine samples

ab156904 is suitable for detecting total urinary 5-methylcytosine levels resulting from whole body turnover or degradation of methylated DNA/RNA using urine from humans and animals. The urine samples can be in fresh or frozen form.

In this ELISA-like inhibitory competitive immunoassay assay, urine samples and 5-mC standard are first incubated with a 5-mC antibody solution and then transferred to the wells coated with methylated DNA (5-mC). The well is washed to remove any unbound reagents after incubation and then a detection antibody is added to generate a signal that can be measured fluorometrically by reading the fluorescent intensity in a microplate spectrophotometer. Because 5-mC in the urine sample inhibits the binding of 5-mC antibody to 5-mC coated on the well, higher concentrations of 5-mC

in the urine sample lead to a reduced binding of the antibody to the 5-mC on the strip-well. Therefore the signal or fluorescent intensity measured from the well will be inversely proportional to the amount of 5-mC in the urine sample and the amount of 5-mC in the urine sample can be quantified by a comparison with a predetermined 5-mC standard.

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
Binding Solution	5 mL	10 mL	RT
5-mC Standard, 4 µg/mL	10 µL	20 µL	–20°C
Negative Control, 50 µg/mL	10 µL	20 µL	–20°C
320X 5-mC DNA Solution	15 µL	30 µL	–20°C
1000X Capture Antibody	4 µL	8 µL	4°C
2000X Detection Antibody	4 µL	8 µL	–20°C
Enhancer Solution	4 µL	8 µL	–20°C
Fluoro Developer	10 µL	20 µL	–20°C
Fluoro Enhancer	10 µL	20 µL	4°C
Dilution Buffer	4 mL	8 mL	RT
Assay Plate	1 (6 strips)	1 (12 strips)	4°C
Sample Preparation Plate	1 (6 strips)	1 (12 strips)	RT

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Adjustable pipette
- Aerosol resistant pipette tips
- Microplate reader capable of reading fluorescence at Ex/Em = 530/590 nm.
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Plate seal or Parafilm M
- Distilled water
- 1X TE buffer pH 7.5 to 8.0
- Urine sample

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 5-mC DNA Solution

Dilute 1 µL of 320X 5-mC DNA Solution with 320 µL of Binding Solution to make 1X 5-mC DNA Solution. About 80 µL of 1X 5-mC DNA Solution will be required for each assay well.

9.3 1X Capture Antibody

Dilute 1000X Capture Antibody with 1X Wash Buffer at a ratio of 1:1000 (e.g. add 1 µL of 1000X Capture Antibody to 1000 µL of 1X Wash Buffer). About 50 µL of 1X Capture Antibody will be required for each assay well.

9.4 1X Detection Antibody

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

9.5 Diluted Enhancer Solution

Dilute 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.

9.6 **Fluorescence Development Solution**

Add 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer to every 500 µL of Dilution Buffer.

***Note**: Keep each diluted solution, 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.*

10. SAMPLE PREPARATION

Input Urine Volume: Urine amount can range from 0.5-5 µL per assay. An optimal amount is 1 µL per assay. Clear urine samples can be directly used for the assay. Centrifugation at 2500-3000 g for 10 minutes should be required for the samples containing precipitates. Urine sample should be stored at -20°C immediately after collection.

11. STANDARD PREPARATION

Suggested Standard Curve Preparation:

11.1 First, dilute 5-mC Standard to 2 ng/µL (e.g. 1 µL of 5-mC Standard + 1 µL of 1X TE).

11.2 Then, further prepare five different concentrations with the 2 ng/µL diluted 5-mC Standard and 1X TE into 0.05, 0.1, 0.2, 0.4, and 1 ng/µL according to the following dilution chart:

Tube	5-mC Standard (2 ng/µL) (µL)	1X TE (µL)	Resulting 5-mC Standard Concentration (ng/µL)
1	1.0	39.0	0.05
2	1.0	19.0	0.10
3	1.0	9.0	0.20
4	1.0	4.0	0.40
5	1.5	1.5	1.00

Note: *Keep each of the diluted solutions on ice until use any remaining diluted solutions should be discarded if not used within the same day.*

12. PLATE PREPARATION

Sample Preparation Plate: The suggested sample preparation plate (U-shaped bottomed wells) setup in a 48-assay format depicted below (for a 96-assay format, Strips 7 to 12 can be configured as Sample Wells). 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	Negative Control	Negative Control	Sample	Sample	Sample	Sample
C	5-mC Standard 0.05 ng	5-mC Standard 0.05 ng	Sample	Sample	Sample	Sample
D	5-mC Standard 0.1 ng	5-mC Standard 0.1 ng	Sample	Sample	Sample	Sample
E	5-mC Standard 0.2 ng	5-mC Standard 0.2 ng	Sample	Sample	Sample	Sample
F	5-mC Standard 0.4 ng	5-mC Standard 0.4 ng	Sample	Sample	Sample	Sample
G	5-mC Standard 1.0 ng	5-mC Standard 1.0 ng	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

Assay Plate (After Transfer): Use the same plate layout as the Sample Preparation Plate using the flat bottomed welled Assay Plate. **F**or a 96-assay format, strips 7 to 12 can be configured as Sample Wells. The controls and samples can be measured in duplicate.

13. ASSAY PROCEDURE

- Internal Control: Both a negative control and a positive 5-mC standard are provided in this kit. A standard curve can be performed (range: 0.05-1 ng). Because 5-mC levels in urine can vary from individual to individual, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated has been validated. This kit will allow the user to quantify an absolute amount of 5-mC and determine the relative turnover states of methylated DNA/RNA of two different urine samples

5-mC Binding

13.1.1 The Assay Plate will be required for your experiment. 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 Blank Wells: Add 80 µL of Binding Solution to each blank well.

13.1.3 Negative Control Wells: Add 80 µL of 1X 5-mC DNA Solution to each negative control well.

13.1.4 Standard Wells: Add 80 µL of 1X 5-mC DNA Solution to each standard well.

13.1.5 Sample Wells: Add 80 µL of 1X 5-mC DNA Solution to each sample well.

13.1.6 Cover the Assay Plate with plate seal or Parafilm M and incubate at 37°C for 90 minutes.

13.1.7 Meanwhile, predetermine the number of wells required in the Sample Preparation Plate. Cover remaining unused wells with plate seal and set up sample preparation in the subsequent steps (steps 13.1.8 to 13.1.11).

13.1.8 Blank Wells: Add 50 µL of 1X Capture Antibody and 50 µL of 1X Wash Buffer to each blank well.

13.1.9 Negative Control Wells: Add 50 µL of 1X Capture Antibody, 49 µL of 1X Wash Buffer, and 1 µL of Negative Control to each negative control well.

13.1.10 Standard Wells: Add 50 µL of 1X Capture Antibody, 49 µL of 1X Wash Buffer, and 1 µL of Diluted 5-mC Standard at the different

concentrations from 0.05-1 ng/µL (as shown in Section 11 - Standard Preparation) to each standard well.

13.1.11 Sample Wells: Add 50 µL of 1X Capture Antibody, 49 µL of 1X Wash Buffer, and 1 µL of urine sample to each sample well.

Note: *(1) Assay Plate and Sample Preparation Plate set up for the wells are depicted in Section 12 – Plate Preparation; (2) As the volume of the control and sample DNA is very small (1 µL), to ensure that the control and sample DNA are completely added into the wells, the pipette tip should be put in the solution contained in the well and aspirated in/out 1-2 times.*

13.1.12 Mix solution by gently tilting from side to side or shaking the Sample Preparation Plate several times. Cover the Sample Preparation Plate with plate seal or Parafilm M and incubate at 37°C for 60-90 minutes.

13.1.13 Remove the solution from each well of the Assay Plate after its incubation period. Wash each well three times with 150 µL of the 1X Wash Buffer each time. This can be done by simply pipetting 1X Wash Buffer in and out of the wells.

13.1.14 Carefully transfer the solution from each well of the Sample Preparation Plate to the corresponding well of the Assay Plate (e.g. from strip wells 1A through 1H of Plate 2 to strip wells 1A through 1H of Plate 1). The Sample Preparation Plate will no longer be used from this point forward.

13.1.15 Cover the Assay Plate with a plate seal or Parafilm M and incubate at 37°C for 60 minutes.

13.1.16 Remove the solution from each well.

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

13.2 **5-mC Capture**

13.2.1 Add 50 µL of 1X Detection Antibody to each well, then cover and incubate at room temperature for 30 minutes.

13.2.2 Remove the 1X Detection Antibody solution from each well.

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

13.2.4 Add 50 µL of the Diluted Enhancer Solution to each well, then cover and incubate at room temperature for 30 minutes.

13.2.5 Remove the Diluted Enhancer Solution from each well.

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

13.3 **Signal Detection**

13.3.1 Add 50 µL of Fluorescence Development Solution to each well and incubate at room temperature. Closely monitor for a color change, for about 2-4 minutes away from direct or strong lighting. Fluorescence Development Solution will turn pink in the presence of sufficient methylated products.

13.3.2 Read the relative fluorescence units (RFU) on a fluorescence microplate reader within 2-10 minutes at 530ex/590em nm.

Note: *If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.*

13.3.3 Calculate the amount of 5-mC using the formulae provided in Section 14 - Data Analysis.

14. ANALYSIS

To quantify the amount of 5-mC, first generate a standard curve and plot the RFU values against the amount of 5-mC Standard at each concentration point. Next, determine the slope (RFU/ng) of the standard curve using linear regression of the most linear part (include at least 4 concentration points) of the standard curve for optimal slope calculation.

Calculate the amount and concentration of 5-mC in urine sample using the following formulas:

$$\text{5-mC (ng/mL)} = \frac{(\text{Sample RFU} - \text{Blank RFU}) - (\text{Negative Control RFU} - \text{Blank RFU})}{\text{Slope} \times \text{Urine Volume*}} \times 1000$$

*Volume added at step 13.1.11.

Example calculation:

Average RFU of blank is 800
Average RFU of Negative Control is 12800
Average RFU of sample is 8800
Slope is -6000 RFU/ng
Urine volume is 1 µL

$$\text{5-mC (ng/mL)} = \frac{8000 - 12000}{-6000 \times 1} \times 1000 = 667 \text{ ng/mL}$$

Typical Results

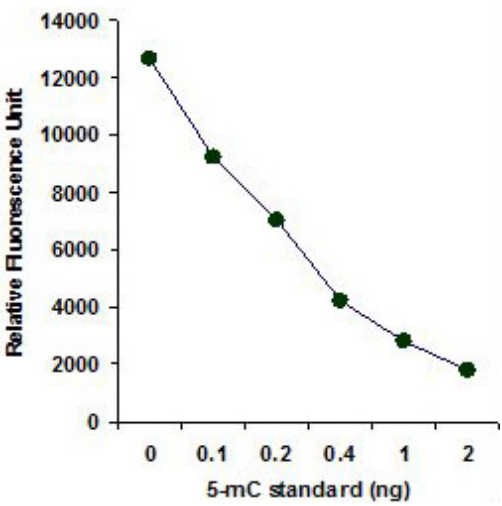


Figure 1. 5-mC standard was added into the assay wells at different concentrations and then measured with Abcam's Urine 5-methylcytosine Quantification Kit (Fluorometric) (ab156904).

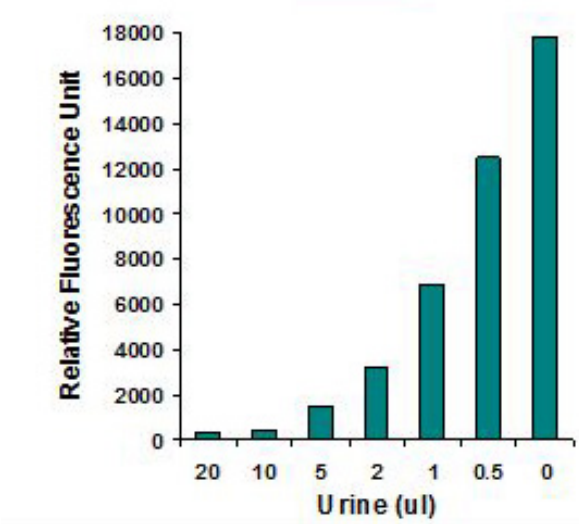


Figure 2. 5-mC level is quantified from different volumes of human urine using Abcam's Urine 5-methylcytosine Quantification Kit (Fluorometric) (ab156904).

15. TROUBLESHOOTING

Problem	Cause	Solution
No signal or weak signal in the negative control wells, the standard wells, and the sample wells	Reagents are added incorrectly	Check if reagents are added in the proper order and if any steps in the protocol may have been omitted by mistake
	Antibody was not correctly added into the designated wells of Plate 2 (Section 12 – Plate Preparation)	Ensure the antibody at a proper dilution is added into the designated wells of the Plate 2 (Section 12 – Plate Preparation)
	The bottom of the well is not completely covered by the Binding Solution	Ensure the solution coats the bottom of the well by gently tilting from side to side or by shaking the plate several times
	Incubation time and temperature are incorrect	Ensure the incubation time and temperatures described in the protocol are followed correctly
	Insufficient 5-mC DNA solution is added into the assay wells	Ensure that a sufficient amount of 5-mc DNA solution is added into the wells
	Incorrect absorbance reading	Check if appropriate wavelength (530ex/590em 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 sample wells	Too much urine sample is used	Ensure the volume of urine samples added into the wells is within the recommended range. Optimal volume is 1 µL
No signal or weak signal in only the standard wells	The 5-mC Standard solution is not properly diluted	Properly dilute the 5-mC Standard solution to the different concentrations according to the dilution chart in Section 11 – Standard Preparation
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 5-mC DNA solution	Ensure the well is not contaminated from adding 5-mC DNA solution accidentally or from using contaminated tips
RFU values in the negative control wells, and some of the standard and sample wells are out of the fluorescent plate reader's range	Over development of fluorescence	Decrease the development time in step 13.3.2. Also, RFU can be re-measured after diluting the solution after fluorescence in the wells with PBS at a 1:5 or 1:10 ratio. The RFU ratio remains unchanged in the designated assay wells
Large variation between replicate wells	Fluorescence reaction is not evenly added due to inconsistency in pipetting time	Ensure Fluoro Developer solution is added at the same time between replicates or otherwise maintain the consistent timing in between each addition of solutions

	Fluorescence reaction is not evenly added due to an inconsistent order of adding solutions	Ensure all solutions, particularly Fluoro Developer 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 volume (e.g. 1 µl) 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 to prevent solutions from sticking to the surface
	Did not sufficiently shake the solutions in the wells evenly after adding sample or positive control at step 13.1	Gently 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

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 User Guide. Mix and centrifuge prior to use

16. NOTES

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