ab117129 – Methylated DNA Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of global DNA methylation status using DNA isolated from any species such as mammalians, plants, fungi, bacteria, and viruses

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

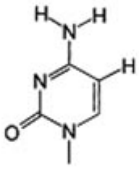
Version 1 Last Updated 31 January 2019

Table of Contents

1. BACKGROUND

DNA methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring by DNA methyltransferases, resulting in 5-methylcytosine (5-mC). In somatic cells, 5-mC is found almost exclusively in the context of paired symmetrical methylation of the dinucleotide CpG, whereas in embryonic stem (ES) cells, a substantial amount of 5-mC is also observed in non-CpG contexts. The biological importance of 5-mC as a major epigenetic modification in phenotype and gene expression has been recognized widely. For example, global decrease in 5-mC content (DNA hypomethylation) is likely caused by methyl-deficiency due to a variety of environmental influences, and has been proposed as a molecular marker in multiple biological processes such as cancer. It has been well demonstrated that the decrease in global DNA methylation is one of the most important characteristics of cancer. Thus, the quantification of 5-mC content or global methylation in cancer cells could provide very useful information for detection and analysis of this disease.

Quite recently, a novel modified nucleotide, 5-hydroxymethylcytosine (5-hmC) has been detected to be abundant in mouse brain and embryonic stem cells. In mammals, it can be generated by oxidation of 5-methylcytosine, a reaction mediated by the Tet family of enzymes and Dnmt proteins. It is a hydroxylated and methylated form of cytosine.



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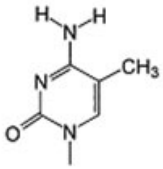
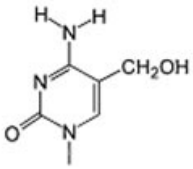


$5^{m}C$



^{hm}C

Unmethylated DNA	Methylated DNA	Hydroxymethylated DNA
T-C-G-T-C-G-A-C-G	T-^{m}C-G-T-^{m}C-G-A-^{m}C-G	T-^{hm}C-G-T-^{hm}C-G-A-^{hm}C-G

The broader functions of 5-hmC in epigenetics are still a mystery today. However, a line of evidence does show that 5-hmC plays a role in DNA methylation structures and patterns.

Because of the presence of both 5-mC and 5-hmC in DNA with possibly different functions, it is important to determine the contents of these two modified nucleotides and their ratios in different cell types and in different compartments of the genome of mammalians. It is particularly important to identify that in healthy and diseased human cell/tissues, the epigenetic change at the DNA level is due to methylation or hydroxymethylation. Several chromatography-based techniques such as HPLC, TLC mass spectrometry are used for detecting 5-mC and 5-hmC. However, these methods are time consuming and have low throughput with high costs.

To address this problem, Abcam offers the Methylated DNA Quantification Kit (Fluorometric) to quantify 5-mC or methylated DNA. This kit is optimized for paired use with our Hydroxymethylated DNA Quantification Kit (ab117130) for simultaneously quantifying both methylated and hydroxymethylated DNA or for quantifying methylated DNA by itself.

The kit has the following advantages and features:

- Fluorometric assay with easy-to-follow steps for convenience and speed. The whole procedure can be finished within 4 hours
- 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
- High sensitivity, of which detection limit can be as low as 50 pg of methylated DNA
- Optimized antibody and enhancer solutions allow high specificity to 5-mC, with no cross-reactivity to unmethylated cytosine and no or negligible cross-reactivity to hydroxymethylcytosine within the indicated concentration range of the sample DNA
- Universal positive and negative controls are included, which are suitable for quantifying methylated DNA from any species

- Strip microplate format makes the assay flexible: manual or high throughput analysis

Abcam's Methylated DNA Quantification Kit (Fluorometric) contains all reagents necessary for the quantification of global DNA methylation. In this assay, DNA is bound to strip wells that are specifically treated to have a high DNA affinity. The methylated fraction of DNA is detected using capture and detection antibody and then quantified fluorometrically by reading the RFU (relative fluorescence units) with a fluorescence spectrophotometer. The amount of methylated DNA is proportional to the fluorescence intensity measured.

ab117129 is suitable for detecting global DNA methylation status using DNA isolated from any species such as mammals, plants, fungi, bacteria, and viruses in a variety of forms including but not limited to cultured cells, fresh and frozen tissues, paraffin-embedded tissues, plasma/serum samples, and body fluid samples. This kit is particularly suitable for samples only available in small amounts such as laser capture microdissection samples and embryos.

The amount of DNA for each assay can be 20-200 ng. For optimal quantification, the input DNA amount should be 100 ng, as methylated DNA varies from tissue to tissue and can be less than 1% of total DNA in some species.

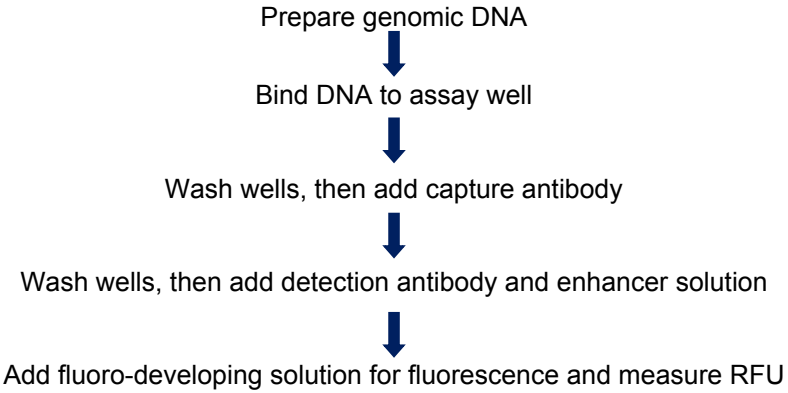
Starting materials can include various tissue or cell samples such as cells from flask or microplate cultured cells, fresh and frozen tissues, paraffin-embedded tissues, plasma/serum samples, body fluid samples, etc.

Both negative and positive DNA controls are provided in this kit. A standard curve can be performed (range: 0.2-10 ng) or a single quantity of methylated DNA can be used as a positive control. Because global methylation can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples at different DNA concentrations to ensure that the signal generated falls within range of the microplate reader used. This kit will

allow the user to quantify an absolute amount of methylated DNA and determine the relative methylation states of two different DNA samples.

To avoid cross-contamination, carefully pipette the sample or solution into the strip wells. Use aerosol barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

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.

Note: *The Negative Control I is an unmethylated polynucleotide containing 50% of cytosine. The Positive Control is a methylated polynucleotide containing 50% of 5-methylcytosine.*

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
Negative Control, 20 µg/mL	10 µL	20 µL	-20°C
Positive Control, 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	8µL	16 µL	-20°C
Enhancer Solution	8 µL	16 µL	-20°C
Fluoro Developer	8 µL	16 µL	-20°C
Fluoro Enhancer	8 µL	16 µL	4°C
Fluoro Dilutor	4 mL	8 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
- Aerosol resistant pipette tips
- Fluorescence microplate reader capable of reading fluorescence at 530 excitation and 590 emission 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
- 1X PBS pH 7.2 to 7.5
- Isolated DNA of interest

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

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.

10. SAMPLE PREPARATION

Input DNA Amount: DNA amount can range from 20-200 ng per reaction. An optimal amount is 100 ng per reaction. Starting DNA may be in water or in a buffer such as TE.

DNA Isolation: You can use your method of choice for DNA isolation. Isolated genomic DNA can be stored at 4°C (short term) or -20°C (long term) until use.

11. STANDARD PREPARATION

Single Point Control Preparation: Dilute the Positive Control with 1X TE to 5 ng/µL (e.g. 1 µL of Positive Control + 3 µL of 1X TE).

Suggested Standard Curve Preparation:

11.1 First, dilute the Positive Control to 10 ng/µL (e.g. 5 µL of Positive Control + 5 µL of 1X TE).

11.2 Then, further prepare five different concentrations with the 10 ng/µL diluted Positive Control and 1X TE into 0.5, 1, 2, 5, and 10 ng/µL according to the following dilution chart:

Tube	Positive Control (10 ng/µL) (µL)	1X TE (µL)	Resulting Positive Control Concentration (ng/µL)
1	1.0	19.0	0.5
2	1.0	9.0	1.0
3	1.0	4.0	2.0
4	2.5	2.5	5.0
5	4.5	0.0	10.0

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

Single Point Positive Control: The suggested strip-well plate setup using a single point positive control in a 48-assay format. The controls and samples can be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Negative Control	Negative Control	Sample	Sample	Sample	Sample
B	Positive Control	Positive Control	Sample	Sample	Sample	Sample
C	Sample	Sample	Sample	Sample	Sample	Sample
D	Sample	Sample	Sample	Sample	Sample	Sample
E	Sample	Sample	Sample	Sample	Sample	Sample
F	Sample	Sample	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

Standard Curve: The suggested strip-well plate setup for standard curve preparation in a 48-assay format. The controls and samples can be measured in duplicate.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	Negative Control	Negative Control	Sample	Sample	Sample	Sample
B	Positive Control 0.5 ng	Positive Control 0.5 ng	Sample	Sample	Sample	Sample
C	Positive Control 1.0 ng	Positive Control 1.0 ng	Sample	Sample	Sample	Sample
D	Positive Control 2.0 ng	Positive Control 2.0 ng	Sample	Sample	Sample	Sample
E	Positive Control 5.0 ng	Positive Control 5.0 ng	Sample	Sample	Sample	Sample
F	Positive Control 10.0 ng	Positive Control 10.0 ng	Sample	Sample	Sample	Sample
G	Sample	Sample	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

13. ASSAY PROCEDURE

13.1 DNA Binding

13.1.1 Predetermine the number of strip wells 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 Add 80 µL of Binding Solution to each well.

13.1.3 Add 1 µL of Negative Control, 1 µL of Diluted Positive Control (see note below), and 100 ng of your Sample DNA (1-8 µL) into the designated wells depicted in 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: *(1) For a single point control, add 1 µL of the Positive Control at a concentration of 5 ng/µL, as prepared in Section 11 – Standard Preparation; For the standard curve, add 1 µL of Diluted Positive Control at concentrations of 0.5-10 ng/µL (see Section 12 – Plate Preparation). The final amounts should be 0.5, 1, 2, 5, and 10 ng per well. (2) For optimal binding, sample DNA volume added should not exceed 8 µL.*

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 Binding Solution from each well. Wash each well with 150 µL of the 1X Wash Buffer each time for three times.

13.2 Methylated DNA Capture

13.2.1 Dilute the Capture Antibody (at 1:1000 ratio) with 1X Wash Buffer.

13.2.2 Add 50 µL of the Diluted Capture Antibody to each well, then cover and incubate at room temperature for 60 minutes.

13.2.3 Remove the Diluted Capture Antibody solution from each well.

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

13.2.5 Dilute the Detection Antibody (at 1:2000 ratio) with 1X Wash Buffer.

13.2.6 Add 50 µL of the Diluted Detection Antibody to each well, then cover and incubate at room temperature for 30 minutes.

13.2.7 Remove the Diluted Detection Antibody solution from each well.

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

13.2.9 Dilute the Enhancer Solution (at 1:5000 ratio) with 1X Wash Buffer.

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

13.2.11 Remove the Diluted Enhancer Solution from each well.

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

13.2.13 Wash each well with 150 µL of the 1X PBS one time.

13.3 **Signal Detection**

13.3.1 Prepare Fluoro-Development Solution by adding 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer into each 500 µL of Fluoro Dilutor.

13.3.2 Add 50 µL of Fluoro-Development Solution into each well and incubate at room temperature for 1-4 minutes away from light. The color in the standard wells containing the higher concentrations may turn pink during this period. Measure and read RFU (relative fluorescence units) on a fluorescence microplate reader at Ex/Em = 530/590 nm.

Note: *If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate and read the RFU on a fluorescence microplate reader at Ex/Em = 530/590 nm.*

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

14. ANALYSIS

To determine the relative methylation status of two different DNA samples, simple calculation of percentage of 5-hmC in total DNA can be carried out using the following formula:

$$\text{5-mC \%} = \frac{(\text{Sample RFU} - \text{Negative Control RFU}) \div S}{(\text{Positive Control RFU} - \text{Negative Control RFU}) \times 2^{*} \div P} \times 100\%$$

S is the amount of input sample DNA in ng.
P is the amount of input Positive Control in ng.
*5 is a factor to normalize 5-mC in the Positive Control to 100%, as the Positive Control contains only 50% of 5-mC.

Example calculation:

Average RFU of Negative Control is 1000
Average RFU of Positive Control is 31000
Average RFU of sample is 21000
S is 100 ng
P is 5 ng

$$\text{5-mC \%} = \frac{(21000 - 1000) \div 100}{(31000 - 1000) \times 2 \div 5} \times 100\% = 1.67\ \%$$

To quantify the absolute amount of methylated DNA using an accurate calculation, first generate a standard curve and plot the RFU values versus the amount of Positive Control at each concentration point. Next, determine the slope (RFU/ng) of the standard curve using linear regression using the

most linear part (include at least 4 concentration points) of the standard curve for optimal slope calculation. Now calculate the amount and percentage of methylated DNA (5-mC) in total DNA using the following formulae:

$$\text{5-mC (ng)} = \frac{\text{Sample RFU} - \text{Negative Control RFU}}{\text{Slope} \times 2^{*}}$$

$$\text{5-mC (\%)} = \frac{\text{5-mC Amount (ng)}}{S} \times 100\%$$

S is the amount of input sample DNA in ng.
*2 is a factor to normalize 5-mC in the Positive Control to 100%, as the Positive Control contains only 50% of 5-mC.

Example calculation:

Average RFU of Negative Control is 1000
Average RFU of sample is 21000
Slope is 6000 RFU/ng
S is 100 ng

$$\text{5-mC (ng)} = \frac{21000 - 1000}{6000 \times 2} = 0.167 \text{ ng}$$

$$\text{5-mC (\%)} = \frac{1.67}{100} \times 100\% = 1.67\%$$

15. TROUBLESHOOTING

Problem	Cause	Solution
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
	The well is incorrectly washed before DNA binding.	Ensure the well is not washed prior to adding the positive control and sample
	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 shaking the plate several times
	Incubation time and temperature are incorrect	Ensure the incubation time and temperature described in the protocol is followed correctly
	Insufficient input materials	Ensure that a sufficient amount of positive control (> 1 ng) and samples (>100 ng) is added into the wells
	Incorrect fluorescence reading	Check if appropriate fluorescence wavelength (Ex/Em = 530/580 nm) is used
No signal or weak signal in both the positive control and sample wells	Kit was not stored or handled properly	Ensure all components of the kit were stored at the appropriate temperature and caps are tightly capped after each opening or use

No signal or weak signal in only the standard curve wells	The positive control DNA is insufficiently added to the well in step D3	Ensure a sufficient amount of positive control DNA is added
	The Positive Control is degraded due to improper storage conditions	Follow the Storage and Stability guidance of this User Guide for storage of the Positive Control
High Background Present for the Negative control	Insufficient washing of wells	Check if washing recommendations at each step is performed according to the protocol
	Contaminated by sample or positive control	Ensure the well is not contaminated from adding sample or standard accidentally or from using contaminated tips
	Incubation time is too long	The incubation time at step 13.1.4 should not exceed 2 hours
High Background Present for the Negative control	Over development of fluorescence.	Decrease the development time in step 13.3.2

16. NOTES

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