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ab117128 – Methylated DNA Quantification Kit (Colorimetric)

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.

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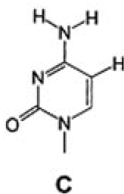
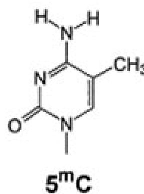
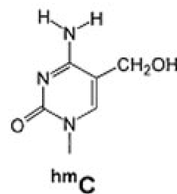
1. BACKGROUND

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.







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 (Colorimetric) 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.

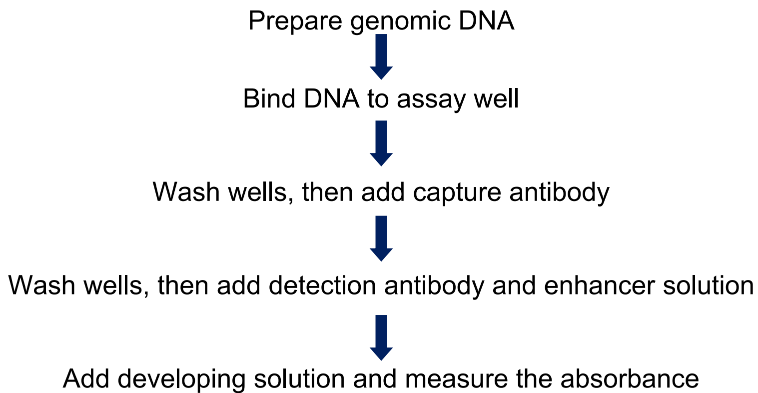
This kit has the following advantages and features:

- Colorimetric assay with easy-to-follow steps for convenience and speed. The entire 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 the detection limit can be as low as 0.2 ng of methylated DNA (methylation).
- 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-well microplate format makes the assay flexible: manual or high throughput analysis

The Methylated DNA Quantification Kit (Colorimetric) 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 antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. The amount of methylated DNA is proportional to the OD intensity measured.

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 upon receipt.

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

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

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
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

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

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Adjustable pipette, multi-channel recommended
- 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
- 1X TE buffer pH 7.5 to 8.0
- 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

Prepare fresh reagents immediately prior to use.

9.1 **1X Wash Buffer**

Add 13 mL of 10X Wash Buffer to 117 mL of distilled water (pH 7.2-7.5). Diluted 1X Wash Buffer can now be stored at 4°C for up to six months.

9.2 **Positive Control**

9.2.1 **Single Point Control Preparation.**

Dilute Positive Control with 1X TE to 5 ng/µL (1 µL of Positive Control + 3 µL of TE)

9.2.2 **Suggested Standard Curve Preparation.**

First, dilute Positive Control to 10 ng/µL (5 µL of Positive Control + 5 µL of 1X TE). 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 (µL)	1X TE (µL)	Final Conc (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	4.0	0.0	10.0

10. SAMPLE PREPARATION

10.1 **Input DNA Amount**: DNA amount can range from 50 ng to 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.

10.2 **DNA Isolation**: You can use your method of choice for DNA isolation.

10.3 **DNA Storage:** Isolated genomic DNA can be stored at 4°C (short term) or -20°C (long term) until use.

11. ASSAY PROCEDURE

11.1 DNA Binding

11.1.1 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).

11.1.2 Add 80 µL of Binding Solution to each well.

11.1.3 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 Table 1 or Table 2. 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 Positive Control at a concentration of 5 ng/µL, as prepared in Step 2; For the standard curve, add 1 µL of Diluted Positive Control at concentrations of 0.5 to 10 ng/µL (see the chart in Step 9.2.2). 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.

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

11.1.5 Remove the Binding Solution from each well. Wash each well with 150 µL of 1X Wash Buffer (diluted from 10X Wash Buffer) three times.

Table 1. 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

Table 2. Standard Curve Preparation. 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

11.2 **Methylated DNA Capture**

11.2.1 Dilute Capture Antibody (at 1:1000 ratio) with the Diluted Wash Buffer.

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

11.2.3 Remove the Diluted Capture Antibody solution from each well.

11.2.4 Wash each well with 150 µL of the Diluted Wash Buffer each time for three times.

11.2.5 Dilute Detection Antibody (at 1:2000 ratio) with the Diluted Wash Buffer.

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

11.2.7 Remove the Diluted Detection Antibody solution from each well.

11.2.8 Wash each well with 150 µL of the Diluted Wash Buffer each time for four times.

11.2.9 Dilute Enhancer Solution (at 1:5000 ratio) with the Diluted Wash Buffer.

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

11.2.11 Remove the Diluted Enhancer Solution from each well.

11.2.12 Wash each well with 150 µL of the Diluted Wash Buffer each time for five times.

11.3 **Signal Detection**

11.3.1 Add 100 µL of Developer 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 Developer Solution solution will turn blue in the presence of sufficient methylated DNA.

11.3.2 Add 50 µLof 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 at 450 nm within 2 to 15 min.

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

12. ANALYSIS

12.1 Relative Quantification of 5-mC

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

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

S is the amount of input sample DNA in ng.
P is the amount of input positive control 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 OD_{450} of Negative Control is 0.075
Average OD_{450} of Positive Control is 0.675

Average OD_{450} of Sample is 0.475
S is 100 ng
P is 5 ng

$$5\text{-mC } \% = \frac{(0.475 - 0.075) \div 100}{(0.675 - 0.075) \times 2 \div 5} \times 100\% = 1.67\%$$

12.2 **Absolute Quantification of 5-mC**

To quantify the absolute amount of methylated DNA using an accurate calculation, first generate a standard curve and plot the OD values versus the amount of Positive Control at each concentration point. Next, determine the slope (OD/ng) of the standard curve using linear regression (Microsoft Excel's linear regression functions are suitable for such calculation) and 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 formulas:

$$\text{5-mC (ng)} = \frac{\text{(Sample OD – Negative Control OD)}}{\text{Slope x 2*}}$$

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

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 OD450 of Negative Control is 0.075

Average OD450 of Sample is 0.475

Slope is 0.12 OD/ng

S is 100 ng

5-mC (ng)=

$$\frac{(0.475 - 0.075)}{0.12 \times 2} = 1.67 \text{ ng}$$

5-mC % =

$$\frac{1.67}{100} \times 100\% = 1.67\%$$

13. 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

Problem	Cause	Solution
No signal or weak signal in both the positive control and sample wells	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 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 11.1.3	Ensure a sufficient amount of positive control DNA is added.
	The Positive control is degraded due to improper storage conditions.	Follow the Storage guidance of this User Guide for storage of Positive Control.
High Background Present for the Negative control	Insufficient washing of wells.	Check if washing recommendations at each step are 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 11.1.4 should not exceed 2 hours.
High Background Present for the Negative control	Over development of color.	Decrease the development time in Step 11.3.1 before adding Stop Solution in Step 11.3.2.

14. NOTES

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