ab117131 – Hydroxymethylated DNA Quantification Kit (Fluorometric)

Instructions for Use

For the measurement of global DNA hydroxymethylation status using DNA isolated from any species

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

Version 3b, Last Updated 18 June 2024

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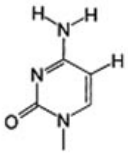
1. BACKGROUND

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.



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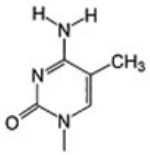
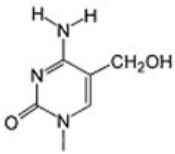


$5^{m}C$



^{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 demethylation, chromatin remodeling, and gene expression regulation, specifically in brain-specific gene regulation:

Conversion of 5-mC to 5-hmC greatly reduced the affinity of MBD proteins to methylated DNA.

The observation that formation of 5-hmC by oxidative damage or by addition of aldehydes via DNMTs prevents DNMT-mediated methylation of the target cytosine.

5-hmC may recruit specific binding proteins that alter chromatin structure or DNA methylation patterns.

5-hmC accounts for roughly 40 percent of the methylated cytosine in Purkinje cells and 10 percent in granule neurons.

This kit has the following advantages and features:

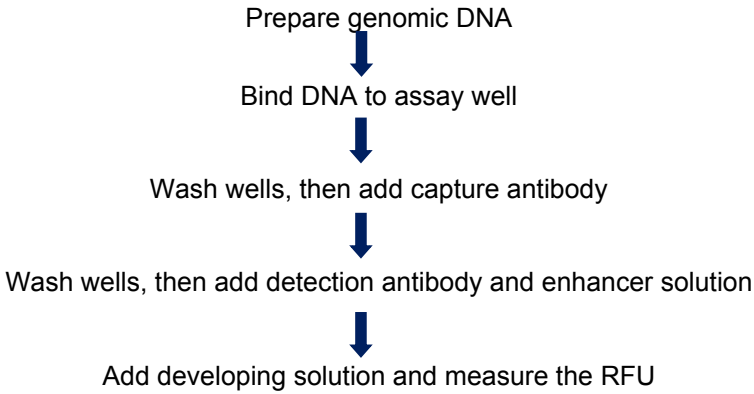
- Fluorometric assay with easy-to-follow steps for convenience and speed. The entire procedure can be finished within 3 hours and 20 minutes
- High sensitivity, of which the detection limit can be as low as 10 pg of hydroxymethylated DNA
- High specificity with no cross-reactivity to unmethylated cytosine and methylcytosine. Only hydroxymethylated DNA (5-hmC) is detected
- Universal positive and negative controls are included, which are suitable for quantifying hydroxymethylated DNA from any species.
- Strip-well microplate format makes the assay flexible: manual or high throughput analysis
- Simple, reliable, and consistent assay conditions

The Hydroxymethylated DNA Quantification Kit (Fluorometric) contains all reagents necessary for the quantification of global DNA hydroxymethylation. In this assay, DNA is bound to strip wells that are specifically treated to have a high DNA affinity. The hydroxymethylated 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 hydroxymethylated DNA is proportional to the fluorescence intensity measured.

ab117131 is suitable for detecting global DNA hydroxymethylation 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.

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.

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 I, 20 µg/mL*	10 µL	20 µL	-20°C
Negative Control II, 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

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

Note: *The Negative Control I is an unmethylated polynucleotide containing 20% of cytosine. Negative Control II is a methylated polynucleotide containing 20% of 5-methylcytosine. The Positive Control is a Hydroxymethylated polynucleotide containing 20% of Hydroxymethylcytosine*.

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
- 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
- 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 **Capture Antibody**

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

9.3 **Detection Antibody**

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

9.4 **Fluoro-Development Solution**

Add 1 µL of Fluoro Developer and 1 µL of Fluoro Enhancer into each 500 µL of Fluoro Dilutor.

9.5 **Enhancer Solution**

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

9.6 **Positive Control**

9.6.1 **Single Point Control Preparation.**

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

9.6.2 **Suggested Standard Curve Preparation.**

First, dilute Positive Control to 5 ng/µL (3 µL of Positive Control + 9 µL of 1X TE). Then further prepare five different concentrations with the 5 ng/µL diluted Positive Control and 1X TE into 0.2, 0.5, 1.0, 2.0, and 5.0 ng/µL according to the following dilution chart.

Tube	Positive Control (µL)	1X TE (µL)	Final Conc (ng/µL)
1	0.5	12.0	0.2
2	1.0	9.0	0.5
3	1.0	4.0	1.0
4	2.0	3.0	2.0
5	5.0	0	5.0

10. SAMPLE PREPARATION

10.1 **Input Amount:** DNA amount can range from 20 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. Isolated genomic DNA can be stored at 4°C (short term) or -20°C (long term) until used.

11. 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 I	Negative Control I	Sample	Sample	Sample	Sample
B	Negative Control II	Negative Control II	Sample	Sample	Sample	Sample
C	Positive Control	Positive Control	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 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 I	Negative Control I	Sample	Sample	Sample	Sample
B	Negative Control II	Negative Control II	Sample	Sample	Sample	Sample
C	Positive Control 0.2 ng/µl	Positive Control 0.2 ng/µl	Sample	Sample	Sample	Sample
D	Positive Control 0.5 ng/µl	Positive Control 0.5 ng/µl	Sample	Sample	Sample	Sample
E	Positive Control 1.0 ng/µl	Positive Control 1.0 ng/µl	Sample	Sample	Sample	Sample
F	Positive Control 2.0 ng/µl	Positive Control 2.0 ng/µl	Sample	Sample	Sample	Sample
G	Positive Control 5.0 ng/µl	Positive Control 5.0 ng/µl	Sample	Sample	Sample	Sample
H	Sample	Sample	Sample	Sample	Sample	Sample

12. ASSAY PROCEDURE

12.1 **DNA Binding**

12.1.1 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).

12.1.2 Add 80 µL of Binding Solution to each well.

12.1.3 Add 1 µL of Negative Control I, 1 µL of Negative Control II,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.

Notes: *(1) For a single point control, add 1 µL of Positive Control at a concentration of 5 ng/µL, as prepared in Step 9.66.1; For the standard curve, add 1 µL of Diluted Positive Control at concentrations of 0.2 to 5 ng/µL (see the chart in Step 9.6.2). The final amounts should be 0.2, 0.5, 1, 2, and 5 ng per well.*
(2) For optimal binding, sample DNA volume added should not exceed 8 µL.

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

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

12.2 **Hydroxymethylated DNA Capture**

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

12.2.2 Remove the Diluted Capture Antibody solution from each well.

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

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

12.2.5 Remove the Diluted Detection Antibody solution from each well.

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

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

12.2.8 Remove the Diluted Enhancer Solution from each well.

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

12.3 **Signal Detection**

12.3.1 Add 50 µL of Fluoro-Development Solution into each well and incubate at room temperature for 1 to 4 min 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. ANALYSIS

13.1 **Relative Quantification of 5-hmC**

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:

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

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-hmC in the positive control to 100%, as the positive control contains only 20% of 5-hmC.

Example calculation:
Average RFU of Negative Control II is 900
Average RFU of Positive Control is 30900

Average RFU of Sample is 8900
S is 100 ng
P is 2 ng

$$\text{5-hmC \%} = \frac{(8900 - 900) \div 100}{(30900 - 900) \times 5 \div 5} \times 100\% = 0.107\%$$

12.3 **Absolute Quantification of 5-mC**

To quantify the absolute amount of Hydroxymethylated 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 5-hmC in total DNA using the following formulas:

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

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

S is the amount of input sample DNA in ng.

* 5 is a factor to normalize 5-hmC in the positive control to 100%, as the positive control contains only 20% of 5-hmC.

Example calculation:

Average RFU of Negative Control II is 900

Average RFU of Sample is 8900

Slope is 15000 RFU/ng

S is 100 ng

5-hmC (ng)=

$$\frac{(8900 - 900)}{15000 \times 5} = 0.107 \text{ ng}$$

5-hmC % =

$$\frac{0.107}{100} \times 100\% = 0.107\%$$

Example of Graph:

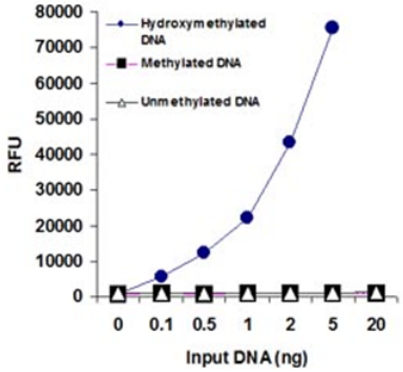


Figure 1. Demonstration of high sensitivity and specificity of 5-hydroxymethylcytosine detection achieved using ab117131. Synthetic unmethylated DNA (contains only cytosine), methylated DNA (contains only 5-methylcytosine), and hydroxymethylated DNA standard (contains only 5-hydroxymethylcytosine) were added into the assay wells at different concentrations and then measured with ab117131.

14. 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 = 530/590 nm) is used

Problem	Cause	Solution
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 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 for storage of 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 D4 should not exceed 2 hours
	Over development of fluorescence	Decrease the development time in Step 11.3.1.

15. NOTES

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)