ab117130 – Hydroxymethylated DNA Quantification Kit (Colorimetric)

Instructions for Use

For 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.

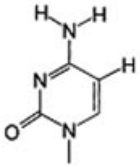
Version 1 Last Updated 1 February 2019

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 widely recognized.

Quite recently, a novel modified nucleotide, 5-hydroxymethylcytosine (5-hmC) has been detected to be abundant in mouse brain and embryonic stem cells. 5-hydroxymethylcytosine was first seen in bacteriophages in 1952. 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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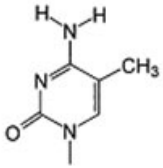
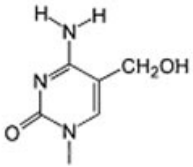


$5^{m}C$



^{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:

1. Conversion of 5-mC to 5-hmC greatly reduced the affinity of MBD proteins to methylated DNA.
2. 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.
3. 5-hmC may recruit specific binding proteins that alter chromatin structure or DNA methylation patterns.
4. 5-hmC accounts for roughly 40 percent of the methylated cytosine in Purkinje cells and 10 percent in granule neurons.

Because of the presence of 5-hmC in DNA with unclear functions in gene regulation and the discovery of the enzymes that produce 5-hmC, it is considered rather important to know the distribution of this base in different cell types and in different compartments of the genome of mammalians. It is particularly important to identify hydroxymethylation status in human cell/tissues with and without diseases. Several chromatography-based techniques such as HPLC and TLC mass spectrometry are used for detecting 5-hmC. However these methods are time consuming and have low throughput with high costs. Currently used methylated DNA analysis methods including restriction enzyme digestion and bisulfite or MeDIP-mediated MS-PCR and sequencing are also not suitable for 5-hmC detection as 5-hmC and 5-mC are virtually indistinguishable with these methods.

To address this problem, Abcam offers the Hydroxymethylated DNA Quantification Kit (Colorimetric) which uses a unique procedure to quantify global DNA hydroxymethylation.

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 3 hours and 45 minutes
- High sensitivity, of which the detection limit can be as low as 40 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

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

ab117130 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.

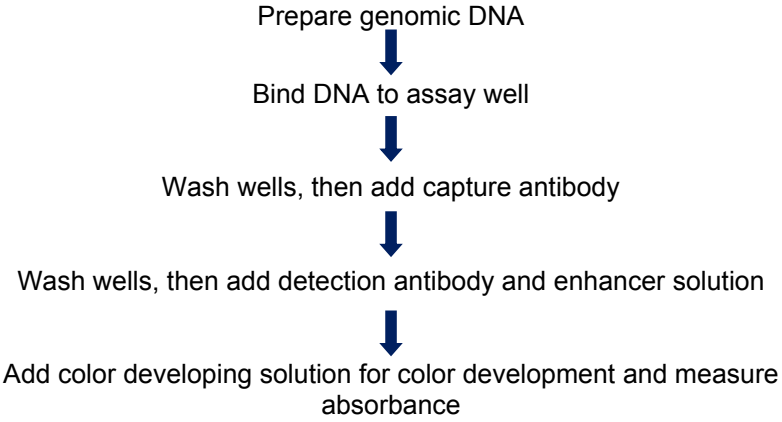
The amount of DNA for each assay can be 50 to 200 ng. For optimal quantification, the input DNA amount should be 200 ng, as hydroxymethylated DNA (hmDNA) is generally less than 0.6% of total DNA.

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: 0.5-10 ng) or a single quantity of hydroxymethylated DNA can be used as a positive control. Because global hydroxymethylation can vary from tissue to tissue, and from normal and diseased states, it is advised to run replicate samples to ensure that the signal generated is validated. This kit will allow the user to quantify an absolute amount of hydroxymethylated DNA and determine the relative hydroxymethylation 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 20% of cytosine. The Negative Control II is a methylated polynucleotide containing 20% of 5-methylcytosine. The Positive Control is a hydroxymethylated polynucleotide containing 20% of hydroxymethylcytosine.*

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
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

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 absorbance at 450 nm
- 1.5 mL microcentrifuge tubes
- Incubator for 37°C incubation
- Plate seal or Parafilm M
- Distilled water
- 1X TE buffer pH 7.5-8.0
- 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

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 50-200 ng per reaction. An optimal amount is 200 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 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 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 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: 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 0.5 ng	Positive Control 0.5 ng	Sample	Sample	Sample	Sample
D	Positive Control 1.0 ng	Positive Control 1.0 ng	Sample	Sample	Sample	Sample
E	Positive Control 2.0 ng	Positive Control 2.0 ng	Sample	Sample	Sample	Sample
F	Positive Control 5.0 ng	Positive Control 5.0 ng	Sample	Sample	Sample	Sample
G	Positive Control 10.0 ng	Positive Control 10.0 ng	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 I, 1 µL of Negative Control II, 1 µL of diluted Positive Control (see note below), and 200 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 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 Hydroxymethylated 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 1:1000 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.

13.3 **Signal Detection**

13.3.1 Add 100 µL of Developer Solution to each well and incubate at room temperature for 1-10 minutes away from light. Begin monitoring color change in the sample wells and control wells. The Developer Solution will turn blue in the presence of sufficient hydroxymethylated DNA.

13.3.2 Add 50 µL of 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 the Stop Solution and the absorbance should be read on a microplate reader at 450 nm within 2-15 minutes.

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 % 5-hmC using the formulae provided in Section 14 - Data Analysis.

14. ANALYSIS

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

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

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 OD450 of Negative Control II is 0.085
Average OD450 of Positive Control is 0.780
Average OD450 of sample is 0.158
S is 200 ng
P is 5 ng

$$\text{5-hmC \%} = \frac{(0.158 - 0.085) \div 200}{(0.780 - 0.085) \times 5 \div 5} \times 100\% = 0.0525\ \%$$

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 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 your total DNA using the following formulae:

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

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

S is the amount of input sample 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 OD450 of Negative Control II is 0.085
Average OD450 of sample is 0.158
Slope is 0.14 OD/ng
S is 200 ng

$$\text{5-hmC (ng)} = \frac{0.158 - 0.085}{0.14 \times 5} = 0.104 \text{ ng}$$

$$\text{5-hmC (\%)} = \frac{0.104}{200} \times 100\% = 0.052\%$$

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
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
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 13.1.3	Ensure a sufficient amount of positive control DNA is added
	The Positive Control is degraded due to improper storage conditions	Follow the Storage 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 color	Decrease the development time in step 13.3.1 before adding Stop Solution in step 13.3.2

16. NOTES

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