Version 2 Last updated 13 September 2019

ab233487 Global DNA Hydroxymethylation Assay Kit (5hmc, Colorimetric)

For the measurement of global DNA hydroxymethylation levels in DNA isolated form any species type.

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

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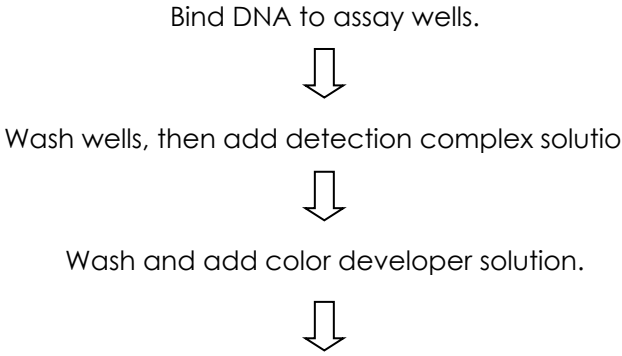
1. Overview

The Global DNA Hydroxymethylation Assay Kit (ab233487) is suitable for detecting global DNA hydroxymethylation levels using DNA isolated from any species including 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.

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 a 5-hmC mAb-based detection complex in a one-step manner and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer.



2. Materials Supplied and Storage

Upon receipt: (1) Store **NC**, **PC**, **SI**, and **ES** at –20°C away from light; (2) store **WB**, **hmAb**, **DS**, and **8-Well Assay Strips** at 4°C away from light; (3) Store **BS** and **SS** at room temperature away from light and check below for storage for individual components. All components of the kit are stable for 6 months from the date of shipment, when stored properly.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

Item	48 well plate	96 well plate	Storage temperature (before prep)	Storage temperature (after prep)
10X Wash Buffer	14 mL	28 mL	4°C	4°C
Binding solution	5 mL	10 mL	RT	RT
Negative Control containing 0% 5-hmC, 50 µg/ml*	50 µL	100 µL	–20°C	–20°C
Positive Control containing 1% 5-hmC, 50 µg/ml*	10 µL	20 µL	–20°C	–20°C
5-hmC Antibody, 1000X*	5 µl	10 µl	4°C	4°C
Signal Indicator, 1000X)*	5 µl	10 µl	–20°C	–20°C
Enhancer Solution, 1000X*	5 µl	10 µl	–20°C	–20°C
Developer Solution	5 ml	10 ml	4°C	4°C
Stop Solution	5 ml	10 ml	RT	RT
8-Well Assay Strips (With Frame)	6	12	4°C	4°C

* Spin the solution down to the bottom prior to use.

3. Materials Required, Not Supplied

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

- Adjustable 8-channel 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 to 8.0
- Isolated DNA of interest.

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

5.1 Diluted Wash Buffer

1. For a 48 reaction size kit, prepare Diluted WB (1X Wash Buffer) by adding 13 ml of WB (10X Wash Buffer) to 117 ml of distilled water and adjust pH to 7.2-7.5.
2. For the 96-reaction size kit, add 26 ml of WB (10X Wash Buffer) to 234 ml of distilled water and adjust pH to 7.2-7.5.

ΔNote: This Diluted WB can now be stored at 4°C for up to six months.

5.2 Binding Solution

1. Ready to use as supplied.

5.3 Negative control

1. Ready to use as supplied.

5.4 Positive control

1. Ready to use as supplied.

5.5 5-hmC Antibody

1. Ready to use as supplied.

5.6 Signal indicator

1. Ready to use as supplied.

5.7 Enhancer Solution

1. Ready to use as supplied.

5.8 Developer solution

1. Ready to use as supplied.

5.9 Stop solution

1. Ready to use as supplied.

5.10 5-hmC Detection complex

1. In each 1 mL of Diluted WB add 1 µL of hmAb, mix and then add 1 µL of Signal indicator and 1 µL of Enhancer solution. Mix well.

Reagents	1 well	8 wells (1 strip)	16 wells (2 strips)	48 wells (6 strips)	96 wells (12 strips)
Diluted WB	2.5 ml	20 ml	40 ml	120 ml	240 ml
BS	100 µl	800 µl	1600 µl	4800 µl	9600 µl
5-hmC Detection Complex	50 µl	400 µl	800 µl	2400 µl	4800 µl
DS	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
SS	0.1 ml	0.8 ml	1.6 ml	4.8 ml	9.6 ml
NC	N/A	2 µl	2 µl	45 µl	45-90 µl
PC	N/A	N/A	Optional	6 µl	6-12 µl

ΔNote: The anticipated approximate volumes of reagents needed are reflected above for this assay.

6. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

Dilute 1 µl of PC with 9 µl of NC to make Diluted PC. Mix well. Then, prepare 6 concentration points for the control by combining PC, Diluted PC, and NC according to the following chart. Mix well to ensure the accuracy of the concentration.

Control	PC (1.0%)	Diluted PC (0.1%)	NC
0.02% PC/well	0 µL	1 µL	9 µL
0.04% PC/well	0 µL	1 µL	4 µL
0.10% PC/well	0 µL	3 µL	3 µL
0.20% PC/well	1 µL	0 µL	9 µL
0.40% PC/well	1 µL	0 µL	4 µL
1.00% PC/well	3 µL	0 µL	3 µL

ΔNote: The above volumes will be sufficient for one standard curve in duplicate (12 wells total). The PC concentrations are based on per assay well, not per microliter.

7. Sample Preparation

General sample information:

Input DNA should be relatively pure with 260/280 ratio >1.6 and can be diluted with water or TE buffer. The DNA amount can range from 20 ng to 200 ng per reaction. However, we recommend using 100 ng of DNA, which is the optimized input amount for the best results.

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

8. Assay Procedure

ΔNote: Turn on the incubator to 37°C prior to starting the assay so it reaches the required temperature when needed.

8.1 DNA Binding

Review the configuration of the strip-well plate setup for standard curve preparation in a 48-assays format below (for a 96-reaction format, strips 7 through 12 can be configured as Sample). The controls and samples can be measured in duplicate, loaded vertically instead of horizontally.

Well #	Strip 1	Strip 2	Strip 3	Strip 4	Strip 5	Strip 6
A	NC	0.20%PC	Sample 2	Sample 6	Sample 10	Sample 14
B	NC	0.20%PC	Sample 2	Sample 6	Sample 10	Sample 14
C	0.02%PC	0.40%PC	Sample 3	Sample 7	Sample 11	Sample 15
D	0.02%PC	0.40%PC	Sample 3	Sample 7	Sample 11	Sample 15
E	0.04%PC	1%PC	Sample 4	Sample 8	Sample 12	Sample 16
F	0.04%PC	1%PC	Sample 4	Sample 8	Sample 12	Sample 16
G	0.10%PC	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17
H	0.10%PC	Sample 1	Sample 5	Sample 9	Sample 13	Sample 17

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).
2. For negative control wells: Add 100 µl of BS and 2 µl of NC.
3. For positive control wells: Add 100 µl of BS and 2 µl of PC at different concentrations (0.02%-1%) to generate a standard curve (see note below).
4. For sample wells: Add 100 µl of BS and 100 ng of your sample DNA (2-4 µl).

ΔNote: 1) To reduce cross variation between replicates, it is important to load the wells in vertical formation according to the plate layout depicted above. (2) For the positive controls, total DNA is 100 ng per well with different methylation percentages (0.1%, 0.2%, 0.5%, 1%, 2%, and 5%). The positive controls should be assayed in parallel with the samples in the same plate and a new positive control standard curve should be generated for each assay.

ΔNote: 1) For optimal binding and to reduce pipetting error, sample DNA volume added should be 2 µl or more, but should not exceed 5 µl. If the sample DNA is not 100 ng per well, the amount of positive control DNA should be adjusted accordingly to be equal to the amount of the sample DNA that is used to ensure the accuracy of 5-hmC quantification. 2) To ensure that NC, PC, and sample DNA are completely added into the wells, the DNA should be mixed well before use and the pipette tip should be placed into the BS solution in the well and aspirated in/out 1-2 times. Changing the tips each time when adding the sample will increase sample volume accuracy added into each well.

5. Mix solution by gently tilting from side to side or by gently shaking the plate several times to ensure the solution coats the bottom of the well evenly. Cover strip plate with plate seal or Parafilm M and incubate at 37°C for 60 minutes.
6. During the last 10 minutes of sample incubation, prepare the 5-hmC Detection Complex Solution: In each 1 ml of Diluted WB add 1 µl of hmAb, mix and then add 1 µl of SI and 1 µl of ES. Mix well.
7. Remove the BS from each well after 60 minute incubation. Wash each well with 150 µl of the Diluted WB each time for three times. This can be done by simply pipetting Diluted WB in and out of the wells.

8.2 Hydroxymethylated DNA Detection and Signal Measurement

1. Add 50 µl of the 5-hmC Detection Complex Solution to each well, then cover and incubate at room temperature for 50 minutes.
2. Remove the 5-hmC Detection Complex Solution from each well.)
3. Wash each well with 150 µl of the Diluted WB each time for five times.
4. Add 100 µl of DS to each well in a column, not row, simultaneously in a vertical fashion with a multi-channel pipette so that replicates are developed at the same time. Gently shake the plate against a flat surface for 5-10 seconds and incubate at room temperature for about 5 minutes. Monitor color development in the sample wells and control wells. After a few minutes, the DS will turn blue in the presence of sufficient

methylated DNA. The color in the NC wells will remain generally unchanged.

5. When the color in the 1% PC wells turns deep blue, stop the enzyme reaction by adding 100 µl of SS to each well in a column, not row, simultaneously in a vertical fashion with a multi-channel pipette so that replicates are stopped at the same time. Mix the solution by gently shaking the plate against a flat surface and wait 1-2 minutes to allow the color reaction to be completely stopped. The color will change to yellow after adding SS and the absorbance should be read on a microplate reader at 450 nm within 2-15 minutes.

   ΔNote: (1) The color development time may vary from 1-10 minutes based on the speed of color change, but is typically 4-5 minutes. (2) If the strip-well plate frame does not fit in the microplate reader, transfer the solution to a standard 96-well microplate.

8.3 5-hmC% Calculation

To calculate percentage of hydroxymethylated DNA, first generate a standard curve and plot the OD values versus the PC at each percentage point. Next, determine the slope (OD/1%) of the standard curve using linear regression (Microsoft Excel can be used) and the most linear part (at least 4 concentration points including 0 point) of the standard curve for optimal slope calculation. Now, calculate the percentage of hydroxymethylated DNA (5-hmC) in total DNA using the following formula:

$$5\text{-}hmC\% = \frac{Sample\ OD - NC\ OD}{Slope \times S} \times 100\%$$

S is the amount of input sample DNA in ng.

Example Calculation:

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Average OD450 of NC is 0.075
Average OD450 of sample is 0.275
Slope is 1.2 OD/1%
S is 100 ng
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$$5\text{-}hmC\% = \frac{0.195 - 0.075}{1.2 \times 100} \times 100\% = 0.1\%$$

ΔNote: 1) The calculated 5-hmC% is 5-hmC/total DNA (A+G+C+T). If the 5-hmC% would be presented as 5-hmC/(5-hmC+C), simply divide the calculated 5-hmC% by cytosine content of the species if it is available. For example, cytosine content is 21% in human DNA, thus 5-hmC/(5-hmC+C) is 0.1% ÷ 0.21 = 0.048%. (2) In the event that the standard curve is flat due to high ODs starting from the lowest %PC or is flat at high %PCs because of a saturated signal intensity due to extended color development time, the 5-mC% can be calculated with logarithmic or polynomial second order regression, respectively (see Step 10. Calculations).

9. FAQs / Troubleshooting

General troubleshooting points are found at
www.abcam.com/assaykitguidelines

10. Calculations

Method 1: 5-hmC% Calculation Using Logarithmic Second Order Regression.

Use this method when the standard curve is flat due to high ODs starting from the lowest %PC.

a. Plot the average OD value on the Y-axis versus the known 5-hmC percentage of each **PC** point on the X-axis.

b. Graph the second order logarithmic curve* (also see "Example Calculation" below) and obtain second order logarithmic regression equation:

$$Y = a \ln(X) + b$$

Here, X = 5-hmC%; Y = <Sample OD>; a is Slope and b is Y-intercept, respectively.

ΔNote: *Microsoft Excel's logarithmic regression function can be used for easy and convenient calculation of 5-mC%.*

c. Calculate 5-hmC% of the samples based on the following equation, derived from the above equation

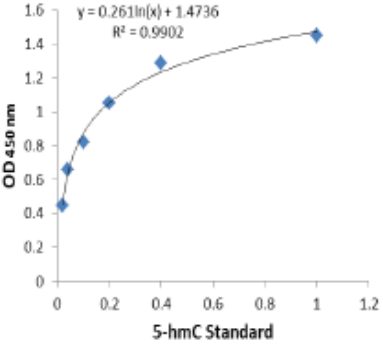
$$5\text{-}hmC\% = e^{[(Y-b)/a]} \div S \times 100\%$$

Here, S is the amount of input sample DNA in ng.

Example calculation:

The example positive control ODs are as follows:

PC%	Mean OD
0.02	0.45
0.04	0.66
0.1	0.82
0.2	1.05
0.4	1.29
1.0	1.45



ΔNote: The average NC OD is 0.06 and the sample OD is 0.591

ΔNote: Standard curve generated with use of the positive controls.

Data was graphed using a Standard Scatter (XY) Chart in Microsoft Excel. In the figure above, a = 0.261, b = 1.4736, Y = 0.591. Input DNA amount was 100 ng.
5-hmC% of the sample was calculated accordingly:

$$5 - hmC\% = e^{[(0.591 - 1.4736) \div 0.291]} \div 100 \times 100\% = 0.03\%$$

Method 2: 5-hmC% Calculation Using Polynomial Second Order Regression.

Use this method when the standard curve is flat due to a saturated signal intensity at high %PCs.

a. Plot the delta OD values (the ODs after subtracting NC ODs) in the Y-axis versus the known 5-hmC percentage of each PC point in the X-axis.

b. Graph the second order polynomial curve* (also see example calculation below) and obtain second order polynomial regression equation:

$$Y = aX^2 + bX$$

Here X = 5-hmC%, Y = sample OD – NC OD, a and b is known slope1 and slope 2, respectively.

ΔNote: Microsoft Excel's polynomial regression function could be used for easy and convenient calculation of 5-hmC%.

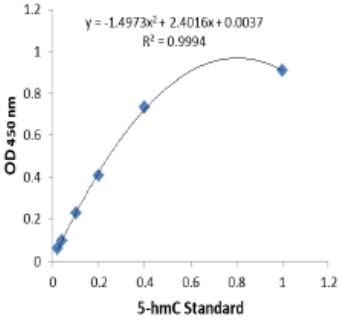
c. Calculate 5-hmC% of the samples based on the following equation, derived from the above equation.

$$5\text{-}hmC\% = \frac{(b^2 + 4aY)^{0.5} - b}{2a} \div S \times 100\%$$

Example calculation:

The example positive control ODs are as follows:

PC%	Mean OD	Delta OD
0.02	0.119	0.059
0.04	0.155	0.096
0.1	0.288	0.228
0.2	0.470	0.410
0.4	0.794	0.734
1.0	0.967	0.907



ΔNote: The average NC OD is 0.06 and the sample OD is 0.262.

ΔNote: Standard curve generated with use of the positive controls.

Data was graphed using a Standard Scatter (XY) Chart in Microsoft Excel. In the figure above, a = −1.4973, b = 2.4016, Y = (0.262−0.06) = 0.202
5-hmC% of the sample was calculated accordingly:

$$5-hmC\% = \frac{[(2.4016)^2 + (4 \times \ -1.4973 \ \times 0.202)]^{0.5} - 2.4016}{2 \times \ -1.4973} \div 100 \times 100\%$$
$$= 0.09\%$$

Problem	Possible Cause	Suggestion
No 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 BS (Binding Solution).	Ensure the solution coats the bottom of the well by gently tilting from side to side or gently shaking the plate several times.
	Incubation time and temperature are incorrect.	Ensure the incubation time and temperature described in the protocol are followed correctly.
	Insufficient input materials.	Ensure that a sufficient amount of positive control and samples are added into the 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 the cap is tightly secure after each opening or use.
No signal or weak signal in only the positive control wells	The PC (Positive Control) DNA is insufficiently added to the well in Step 8.1.3.	Ensure a sufficient amount of positive control DNA is added.
	The PC (Positive Control) is degraded due to improper storage conditions.	Follow the Materials supplied and Storage guidance for storage of Positive Control.

High background present in the negative control wells	Insufficient washing of wells.	Check if washing recommendations at each step are performed according to the protocol.
	Contaminated by sample or positive control DNA.	Ensure the well is not contaminated from adding sample or positive control DNA accidentally or from using contaminated tips.
	Incubation time is too long.	The incubation time at Step 8.2.1 should not exceed 2 hours.
	Over development of color.	Decrease the development time in Step 8.2.4 before adding Stop Solution in Step 8.2.5.
Large variation between replicate wells.	Horizontal positioning of well replicates causes inconsistent delays in pipetting and loading of reagents.	Follow the vertical layout example provided. Ensure loading of reagents is also in vertical order with a multi-channel pipette, especially when adding DS (Developer Solution) and DS (Stop Solution) in Step 8.2.
	Color reaction is not evenly stopped due to an inconsistency in pipetting time or in pipetting volume.	Ensure DS and SS are added at the same time between replicates or otherwise maintains a consistent timing in between each addition of solutions. 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 volumes (e.g., 1 µl) are completely added into the wells. Pay special attention at the steps of adding DNA samples and preparing 5-hmC Detection Complex Solution.
	Color reaction is not evenly stopped due to an inconsistent order of adding solutions.	Ensure all solutions, particularly DS (Developer Solution) and SS (Stop Solution), are added in the same order each time as all other solutions.

	Residue wash buffer in some of wells.	Ensure the wash buffer is completely removed at each wash step.
	Solutions or antibodies were 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 at Step 8.2.4 and Step 8.2.5	Gently and evenly 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.
	Splashing of reagents between wells.	Pipette carefully against the sides of the wells to avoid splashing.
	Temperature variations across the plate.	Ensure plates are evenly and fully covered during incubation steps in a stable temperature environment, away from drafts.
Large variation between sample replicate wells only	Sample DNA is sedimented or uneven prior to loading to wells.	Mix your sample DNA sufficiently and evenly prior to loading it into wells.
hmAb 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 hmAb vial until you restore the correct, intended volume according to the Materials Supplied described in this User Guide. Mix and centrifuge prior to use.

11. Typical Data

Data provided **for demonstration purposes only.**

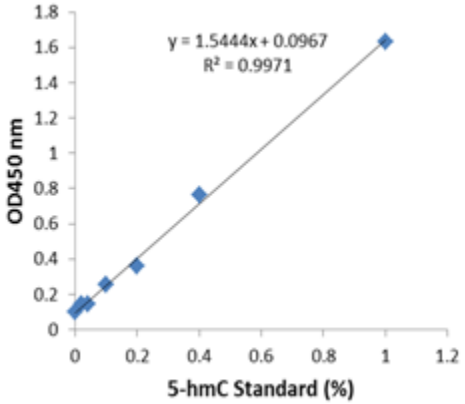


Figure 1: An example of an optimal standard curve generated with 5-hmC standard control.

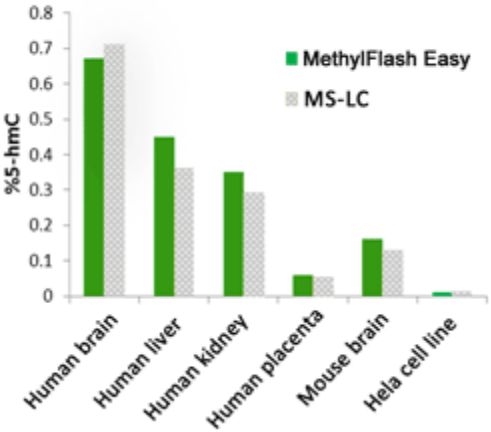


Figure 2: Accurate quantification of 5-hmC content of various DNA samples from different species with the The Global DNA Hydroxymethylation Assay kit (ab233487). The results are closely correlated with those obtained by MS-LC.

12. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | 400 921 0189 / +86 21 2070 0500
Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abc.com | +64-(0)9-909-7829