TM

CpGenome™ 5-hmC Quantitation Kit

Catalog No. 17-10091

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

Methylation of DNA is a well-characterized epigenetic signaling mechanism. In mammalian DNA, methylation occurs at the 5 carbon position on cytosines, often in the context of CpG dinucleotides Changes in DNA methylation are known to have a profound effect on the expression of many eukaryotic genes. Recent studies have shown that 5-mC can be converted to 5-hydroxymethylcytosine (5-hmC) by the TET family of iron-dependent oxygenases. 5-hmC, now referred to as the “sixth base”, is found in all mammalian cells, and relatively high levels have been detected in human brain, mouse brain, and both human and murine embryonic stem cells. Although it is now possible to specifically detect and quantify 5-hmC modifications, little is known about its precise role in epigenetic signaling. Research suggests that methylated DNA may be oxidized to 5-hmC during early gonadal development as a potential mechanism of demethylation in the paternal methylome. Other studies, however, show a very discrete localization of 5-hmC modifications associated specifically with transcription units. The CpGenome™ hmC Quantitation Kit provides a rapid, streamlined, automation-compatible ELISA-based protocol for sensitive quantification of 5-hmC in a wide range of DNA samples.

Overview of CpGenome 5-hmC Quantitation Assay

This assay is designed to determine the levels of 5-hmC DNA in a sample relative to a standard curve. This method is highly sensitive and simple to perform. This solid-phase assay is possible because proteins (antibodies) can be attached to plastics (e.g. a 96-well microtiter plate). The approach used in the CpGenome 5-hmC Quantitation Kit (see figure 1), requires two antibodies that bind to epitopes that do not overlap on the antigen. To utilize this assay, one antibody (the ‘capture’ antibody) is purified and bound to a solid phase (typically a well of a microtiter plate). Antigen is then added and allowed to form a complex with the bound antibody. Unbound products are then removed by washing, and a labeled second antibody (the ‘detection’ antibody) is allowed to bind to the antigen, completing the “sandwich”. The assay is then quantitated by measuring the amount of labeled second antibody bound to the matrix, through the use of a colorimetric substrate. This signal can be compared to a standard curve to estimate the amount of material captured.

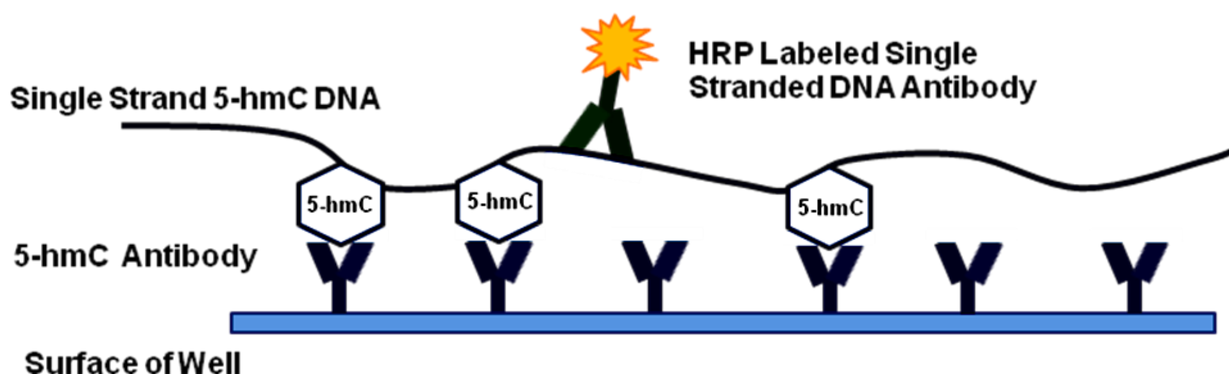

Figure 1: Illustration of CpGenome 5-hmC Quantitation Kit assay principle.

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Kit Overview

The **CpGenome 5-hmC Quantitation Kit** features a high affinity 5-hydroxymethylcytosine antibody which binds 5-hmC–associated genomic DNA isolated from vertebrates, plant, or microbes. This assay is designed for use with intact single-stranded DNA. A set of 5-hmC standards is provided. These standards contain known quantities of 5-hmC, which can be used for either relative quantification of 5-hmC in test samples, or precise quantification of 5-hmC using a standard curve.

Kit Components

The CpGenome 5-hmC Quantitation Kit contains a complete set of reagents to process up to 96 samples in a single experiment. A set of 5-hmC standards**,** comprised of five genomic double-stranded DNA (100 ng/µL) with a specified percentage of 5-hmC, is provided for generating a standard curve or approximating relative levels of 5-hmC in test samples (see Table 2).

Table 1: Reagents List

CpGenome™ 5-hmC Quantitation Kit (Catalog No. 17-10091)			
Part No. 17-10091-1 (2°C to 8°C and Room Temperature Storage)			
Part No.	**Component**	**Quantity**	**Storage Conditions**
CS211803	Plate Coating Buffer	15 mL	2°C to 8°C
CS211802	10X Assay Buffer	30 mL	2°C to 8°C
CS211801	HRP Detection Reagent	15 mL	2°C to 8°C
CS211800	96-well Assay Plate and 8-well Strip Caps	1 plate/12 strips	Room temperature
Part No. 17-10091-2 (-20°C Storage)			
Part No.	**Component**	**Quantity**	**Storage Conditions**
CS211798	5-hmC Antibody (1mg/mL)	25 µL	-20°C Avoid freeze/thaw cycles
CS211799	100x HRP Detection Antibody (1 mg/mL)	100 µL	-20°C Avoid freeze/thaw cycles
CS213071	5-hmC Standard 1 (100 ng/µL)	40 µL	-20°C
CS213059	5-hmC Standard 2 (100 ng/µL)	40 µL	-20°C
CS213060	5-hmC Standard 3 (100 ng/µL)	40 µL	-20°C
CS213061	5-hmC Standard 4 (100 ng/µL)	40 µL	-20°C
CS213062	5-hmC Standard 5 (100 ng/µL)	40 µL	-20°C

Table 2: Percent 5-hmC in DNA Standards

DNA Standards (100 ng/µL)	% 5-hmC
5-hmC Standard 1	0
5-hmC Standard 2	0.03
5-hmC Standard 3	0.12
5-hmC Standard 4	0.23
5-hmC Standard 5	0.55

Required Materials and Equipment Not Supplied

- Sample DNA
- Incubator
- Plate reader (capable of 405 nm detection; e.g. PerkinElmer® Victor™ Plate Reader)
- Multichannel pipettor (recommended)
- Parafilm®, Pipette tips, dilution tubes, and other assorted consumables

Storage Conditions

The CpGenome™ 5-hmC Quantitation Kit is stable for up to 6 months when stored and handled appropriately. Please see the “Kit Components” section for storage conditions for each reagent.

Important Experimental Considerations

Preparation of Sample DNA – The CpGenome™ 5-hmC Quantitation Kit has been optimized for the detection of 5-hmC in purified intact genomic DNA in PBS, Tris-EDTA, or nuclease-free water. DNA samples should not be fragmented by sonication or enzyme-digestion. Detection of 5-hmC using the reagents a protocols provided in this kit requires intact, single stranded, genomic DNA and fragmentation or used of fragmented DNA is not recommended. See protocol below for DNA sample preparation for use with this kit.

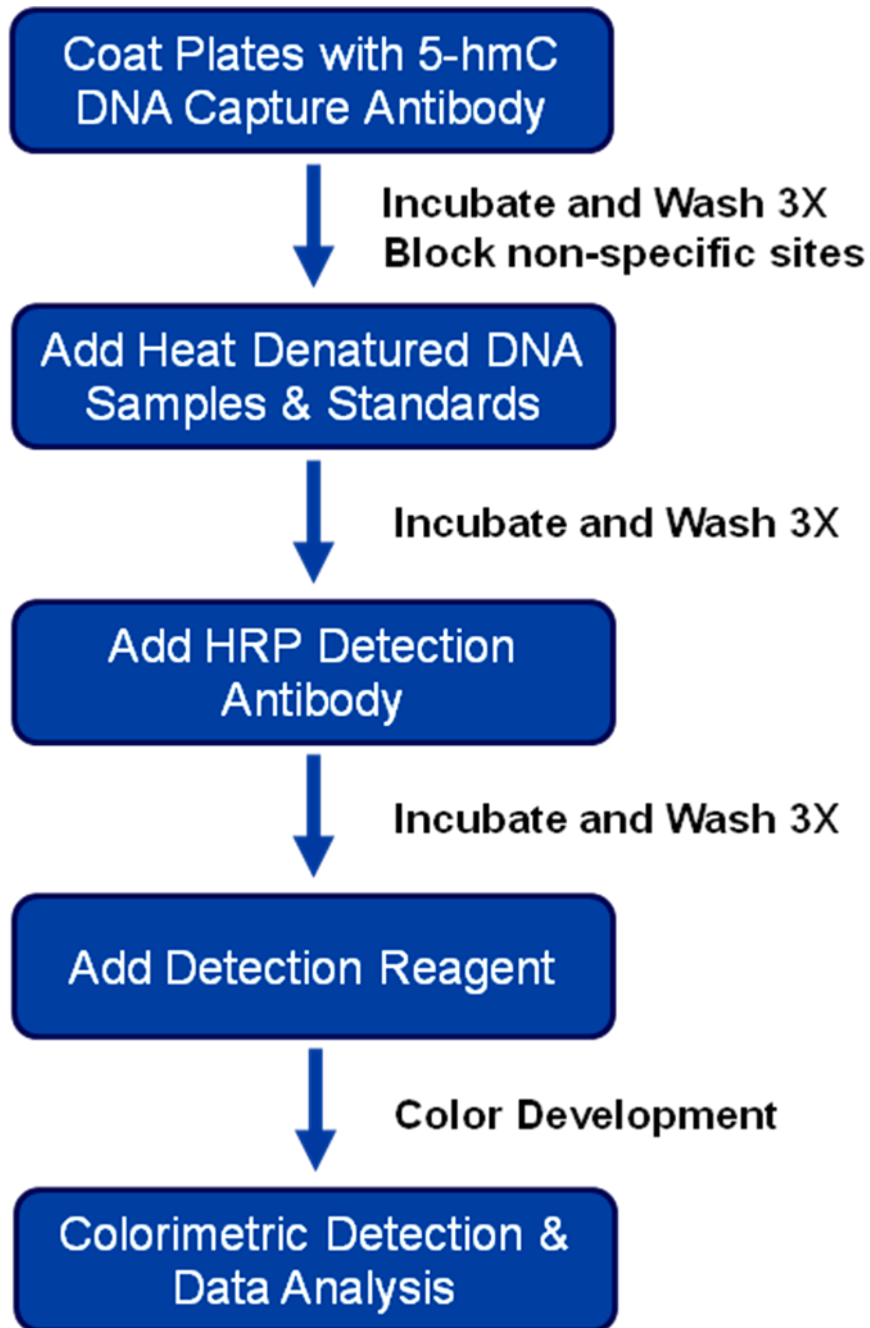
Synthetic DNA – Synthetic DNA samples are not suitable for use with the CpGenome™ 5-hmC Quantitation Kit. Synthetic samples that contain 5-hmC modified bases in high density may prevent the binding of the 5-hmC antibody due to steric hindrance depending on the density of these 5-hmC sites. Standard-curve calculations for these samples will therefore not provide an accurate calculation of 5-hmC levels.

Protocol for DNA Sample Preparation

Sample preparation is a critical step in this assay. The protocol below should be used as a guideline. Sample DNA should not be fragmented by mechanical shearing or enzyme digestion. This has been shown to reduce the absorbance of samples.

1. Dilute the total amount of DNA to make duplicates (225 ng) or triplicates (325 ng) in 100 µL of 1X Assay Buffer using the 0.25 mL PCR tubes.
2. Place tubes in a thermocycler and incubate at 95 °C for 5 minutes.
3. Immediately place tubes on ice and leave for 10 minutes.
4. If performing triplicates, add 225 µL of 1X Assay Buffer to the tube, or 125 µL if performing duplicates to achieve a final concentration of 1ng/µL of DNA.
5. Add 100 µL of diluted samples to each well.
6. Cover the plate with Parafilm and incubate at 37°C for 1 hour.

CpGenome 5-hmC Quantitation Assay Workflow



Protocol

Important Notes

- Please read entire protocol before starting the experiment.
- The protocol is optimized for detection of 5-hmC using 100 ng of single-stranded DNA per well. However, the amount of input DNA can range from 25 to 200 ng per well without influencing the detection and quantification of 5-hmC. To ensure accurate 5-hmC quantification, the amount of DNA per well should be measured precisely.
- All DNA samples must be denatured before using in this experiment. Please see protocol for DNA sample preparation on page 3.
- Approximate levels of 5-hmC in DNA samples can be determined by comparing the absorbance of samples to that of 5-hmC Standard 1 (0% 5-hmC; negative control) and Standard 5 (0.55% 5-hmC; positive control), or any of the DNA standards provided. To precisely quantify levels of 5-hmC in sample DNA, a standard curve should be constructed from the absorbance data for the DNA standards. See the section on "Data Analysis" for step-by-step instructions on how to calculate 5-hmC levels using the Standard curve.
- It is highly recommended that the assay be performed in replicates, for accurate 5-hmC detection. Use a multichannel pipette for "repeat pipetting," if available.
- Ensure that all liquid is removed from the plate wells after each wash step.

A. Buffer Preparation

1. **1X Assay Buffer, pH 7.4:** Prepare a 1:10 dilution of the 10X Assay Buffer. All 30 mL provided can be diluted at once and aliquoted.

B. 5-hmC Quantitation Assay

I. Coating

1. Remove the appropriate number of 8-well strips required to assay samples and 5-hmC Standards. It is recommended that duplicates or triplicates be run for each data point.
2. Prepare a 1ng/µL dilution (1:1,000 dilution) of 1mg/mL 5-hmC antibody in Plate Coating Buffer.
3. Add 100 µL of the 1ng/µL 5-hmC antibody solution to each well. Cover the plate with Parafilm® to prevent evaporation and incubate at 37°C for 1 hour.
   - Incubation can be done for up to 2 hours.

II. Blocking

1. Discard buffer from the wells of the plate.
2. Wash each well with 200 µL of 1X Assay Buffer. Discard liquid; tap each well onto a paper towel to remove any remaining liquid.
3. Repeat Step 2 twice.
4. Add 200 µL of 1X Assay Buffer to each well. Cover the plate with Parafilm and incubate at 37°C for 30 minutes.
   - Incubation can be done for up to 2 hours.

III. Addition and Binding of DNA

1. Place the samples in a thermocycler and incubate at 98°C for 5 minutes to denature the DNA.

    - The DNA must be denatured for detection to occur. Avoid using heat blocks for this step: heat blocks may produce uneven heating and will result in incomplete denaturation of samples. If working with concentrated DNA or small volumes of DNA, dilute input sample in assay buffer prior to denaturing sample.

2. Immediately place samples on ice for 10 minutes. Briefly spin tube to remove any residual liquid from the top of the vial.
3. Dilute single-stranded DNA samples and 5-hmC DNA Standards (supplied at 100 ng/µL) in 1X Assay Buffer to a final concentration of 1ng/µL.
4. Discard buffer from the wells of the plate. Ensure that all liquid is removed from the wells by gently tapping out excess liquid onto a paper towel.
5. Add 100 µL of the diluted samples and 5-hmC Standards to each well. Cover the plate with Parafilm and incubate at 37°C for 1 hour.

IV. Addition of HRP Detection Antibody

1. Discard the buffer from the wells of the plate. Wash each well with 200 µL of 1X Assay Buffer. Ensure that all liquid is removed from the wells by gently tapping out excess liquid onto a paper towel.
2. Repeat Wash in Step 1 twice.
3. Prepare 1:100 final dilution of HRP Detection Antibody in 1X Assay Buffer.

    - For example, add 21 µL of 100X HRP Detection Antibody to 2.1 mL of 1X Assay Buffer. This is sufficient for 20 wells.

4. Add 100 µL of diluted HRP detection antibody mix to each well. Cover the plate with Parafilm and incubate at 37°C for 30 minutes.

V. Colorimetric Detection

1. Discard the buffer from the wells of the plate. Wash each well with 200 µL of 1X Assay Buffer. Ensure that all liquid is removed by the wells by gently tapping out excess liquid onto a paper towel.
2. Add 100 µL of HRP Detection Reagent to each well and allow color to develop at room temperature (20-25°C) for 10 to 60 minutes.
    - Development times can be shorter, if the Detection Reagent is brought to room temperature before adding to the plate.
3. Use a plate reader to measure the absorbance in each well at 405 nm.

VI. Data Analysis

1. To generate a standard curve, plot the absorbance of all 5-hmC standards (y-axis) versus percent 5-hmC (x-axis) and generate a standard curve with your software of choice.
2. Perform a linear regression analysis to obtain the equation of the line.
3. Use the equation of the line to calculate the % 5-hmC in test samples:

$$\%\ 5\text{-hmC}_{\text{test sample}} = \frac{\text{Absorbance (Y)} - \text{Y intercept}}{\text{Slope}}$$

Representative Data

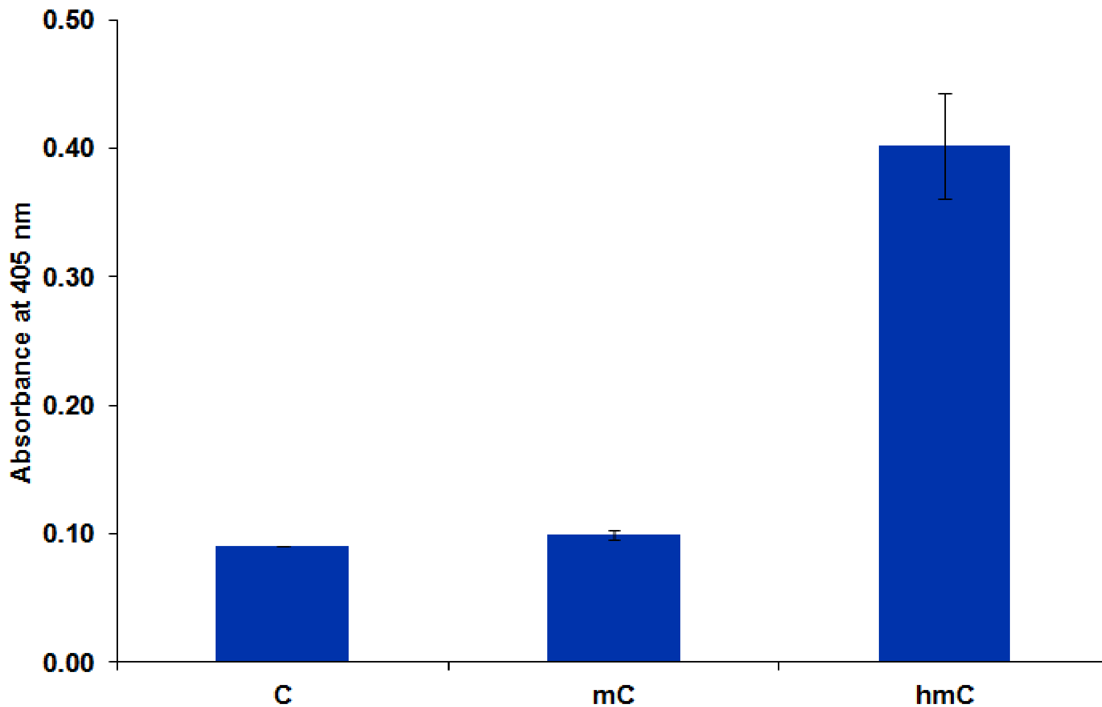


Figure 2: The CpGenome™ 5-hmC Quantitation Kit shows specificity with 5-hmC DNA. Assay was performed as described (in the protocol above using 50 ng of DNA Standards from the CpGenome™ 5-mC and 5-hmC DNA Standard set (Catalog No. S8005). Samples were developed for 30 minutes and absorbance was read at 405 nm.

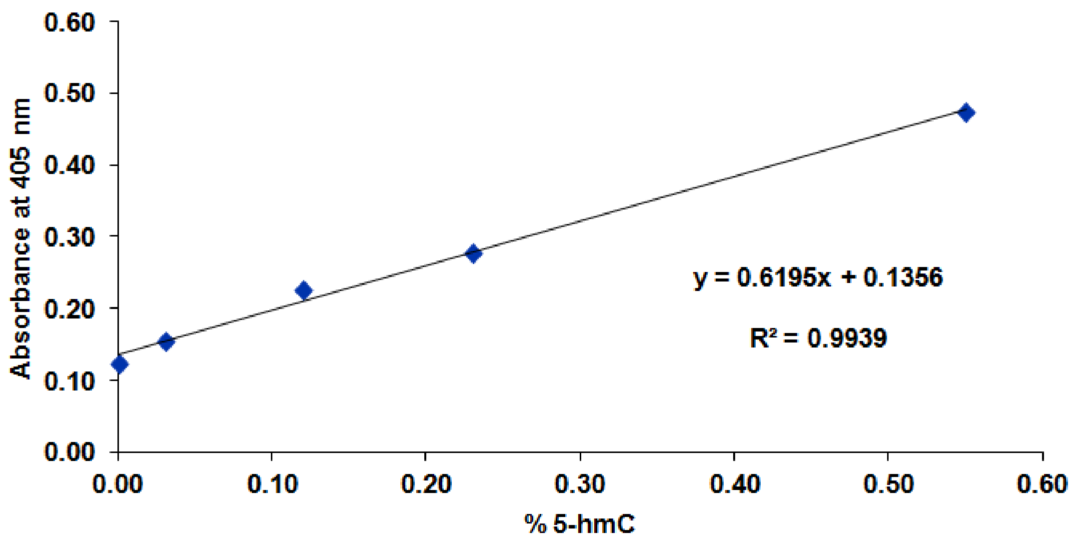


Figure 3: **Representative Standard Curve.** Assay was performed as described in the protocol above using 100 ng of 5-hmC Standards 1 to 5. Samples were developed for 30 minutes and absorbance was read at 405 nm. This standard curve is shown for example only and should not be used for your experiments.

Representative Data Cont.

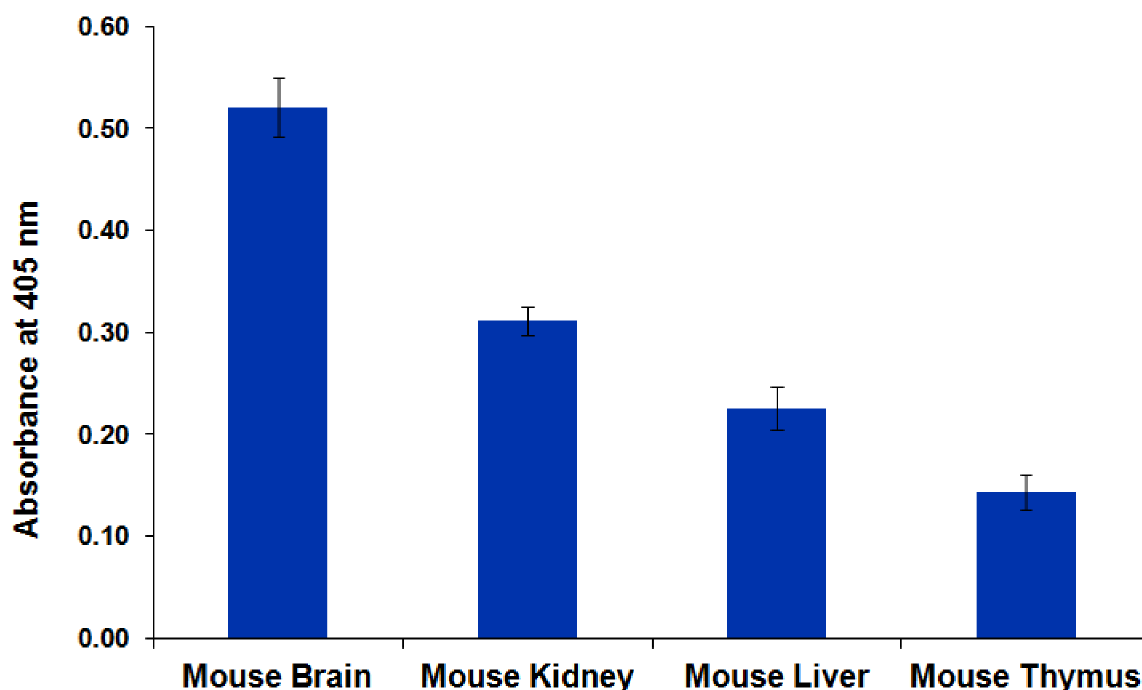


Figure 4: **Performance of CpGenome™ 5-hmC Quantitation Kit with four biological samples.** Assay was performed as described in protocol above using 100 ng of DNA samples that were prepared according to the protocol outlined in the protocol for DNA sample preparation. Samples were developed for 30 minutes and absorbance was read at 405 nm.

References

1. Wu, H., et al. (2011). Genes Dev. 25(23):2436-2452.
2. Ito, S., et al. (2011). Science. 333(6047):1300-1303.
3. Ruzov, A., et al. (2011). Cell Res. 21(9):1332-1342.
4. Ficz, G., et al. (2011). Nature. 473(7347):398-402.

CpGenome 5-hmC Quantitation Assay Troubleshooting

Problem	Possible Cause	Experimental Suggestions
Low or no signal detected for samples or for standards	DNA samples and standards not fully denatured	Make sure thermal cycler is reaching appropriate temperature and samples are immediately chilled on ice as described in protocol.
		Ensure tube surface is making direct contact with thermal cycler block. Avoid using bench top heat blocks for DNA denaturation step: heat blocks may produce uneven heating and incomplete denaturation of samples.
	Use of synthetic DNA samples	Synthetic DNA samples are not suitable for use with the CpGenome™ 5-hmC Quantitation Kit. Synthetic samples may contain 5-hmC clusters which produce steric hindrance and prevent binding of the 5-hmC antibody with all 5-hmC sites.
	Overly fragmented, sheared or degraded DNA	Sample DNA should not be fragmented by mechanical shearing or enzyme digestion. This has been shown to reduce the absorbance of samples.
	Missing or inappropriate addition of reagent.	Carefully follow protocol. Ensure that all reagents are added and appropriately prepared, stored, handled and added at times indicated in protocol.
High variability between replicates	Incomplete removal of buffers from previous steps.	Completely remove all wash buffer and reagents. Gently blot plate on to paper towel to ensure all residual liquid is removed from wells.
	Small input sample volumes	Avoid pipetting small volumes of sample DNA. If DNA is concentrated prepare dilutions in assay buffer prior to denaturing sample. Carefully prepare dilutions using appropriate volumes of input sample and properly calibrated pipettes.
	Cross contamination of samples	Carefully add DNA samples to binding reactions. After incubation of DNA samples on assay plate remove materials prior to first wash.
	Input samples not completely mixed	Ensure all dilutions of input DNA have been mixed by vortexing at least 15 seconds
Weak signal for standard and samples	Temperature of HRP detection reagent	Allow HRP Detection Reagent to warm to room temperature (20-25 °C) before adding to the wells
	Insufficient time allowed for color development.	Increase color development time.
	5-hmC capture antibody coating	Allow sufficient time for plate coating; increase coating time if necessary
	Compromised antibody performance due to improper storage and/or handling	Ensure antibodies are stored at -20 degrees Celsius. Minimize freeze thaw cycles. Aliquot antibodies if partial plates will be run. If antibody performance declines increase the amount of antibody used to 200-400 ng per well (2-4 ng/µL dilution in plate coating step).
Signal is too high	Extended development time	Shorten development time or use less of 5-hmC antibody when coating plates
Standard curve is not linear	Pipetting error or DNA standard not stored appropriately.	Check calibration of pipettes and verify concentration of DNA standard. Mix the DNA samples and measure the standard DNA concentration and adjust to 100 ng/well if needed.

Related Products

Table 3: Products for investigating 5-mC and 5-hmC modifications

Description	Catalog No.
5-hmC Glucosyltransferase	14-1047
CpGenome™ Fast DNA Modification Kit	S7824
CpGenome™ Turbo Bisulfite Modification Kit	S7847
CpG MethylQuest™ DNA Isolation Kit	17-10035
CpG WIZ® BRCA1 Amplification Kit	S7830
CpG WIZ® Oct-4	S7840
CpG WIZ® RASSF1A Amplification Kit	S7813
CpG WIZ® ERα Amplification Kit	S7815
CpG WIZ® Fragile X Amplification Kit	S7807
CpG WIZ® hMLH1 Amplification Kit	S7811
CpG WIZ® GST-pi Amplification Kit	S7808
CpG WIZ® p16 Amplification Kit	S7800
CpG WIZ® p15 Amplification Kit	S7802
CpGenome™ 5-mC & 5-hmC Human DNA Standards	S8003
CpGenome™ 5-mC & 5-hmC Mouse DNA Standards	S8004
CpGenome™ 5mC and 5hmC DNA Standard Set	S8005
CpGenome™ Human Methylated & Non-Methylated DNA Set	S8001
CpGenome™ Human Methylated DNA Standard	S8001M
CpGenome™ Human Non-Methylated DNA Standard	S8001U
CpGenome™ Universal Methylated Mouse DNA Standard	S8000
CpG MethylQuest™ Protein	14-921
Anti-5-Hydroxymethylcytosine, clone AB3/63.3	MABE176
Anti-5-Methylcytosine Mouse, clone 162 33 D3	NA81-50UG
Anti-5-Methylcytosine, clone 33D3	MABE146
Anti-DNA Methyltransferase 1	AB3429
Anti-DNA Methyltransferase 3a	AB3431
Anti-DNA Methyltransferase 3b	AB3433
Anti-DNMT3A2	07-2050
Anti-DNMT3L	ABD78
Anti-Kaiso, clone 6F	05-659
Anti-MBD1, C-terminus	07-2054
Anti-MBD2	07-198
Anti-MBD4	07-2057
Anti-MeCP2 (Rabbit Polyclonal)	07-013
Anti-Methylcytosine Dioxygenase TET1	09-872
Anti-Phospho-DNMT1(Ser714)	07-1594
DNA Methyltransferase Inhibitor	260920
DNA Methyltransferase Inhibitor II, SGI-1027	260921

For a complete list of DNA methylation products, visit www.millipore.com/epigenetics.

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