# Global 5-hmC Quantification

(version A1)

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**TABLE OF CONTENTS** **Page**

**Overview** . . . . . 1

**Flow Chart of Process** . . . . . 2

**Introduction** . . . . . 3

**References** . . . . . 4

**Traditional Methods to Study Hydroxymethylcytosine** . . . . . 5

**Kit Performance and Benefits** . . . . . 6

Global 5-hmC Quantification Assay . . . . . 6

**Kit Components and Storage** . . . . . 7

Additional Materials Required . . . . . 7

**Protocols**

Buffer Preparation and Recommendations . . . . . 8

Quick Chart for Preparing Buffers . . . . . 11

Assay Protocol . . . . . 12

Section A. MseI Digestion of Genomic DNA . . . . . 12

Section B. Plate Set Up . . . . . 13

Section C. Plate Coating . . . . . 13

Section D. Standard Curve Preparation . . . . . 14

Section E. DNA Sample Preparation . . . . . 15

Section F. Binding to the Coated Plate . . . . . 15

Section G. Binding of Primary Antibody . . . . . 15

Section H. Binding of Secondary Antibody . . . . . 16

Section I. Colorimetric Reaction . . . . . 16

Section J. Analysis of Results . . . . . 16

**Appendix**

Section K. Troubleshooting Guide . . . . . 18

Section L. Related Products . . . . . 19

**Technical Services** . . . . . 20

## Overview

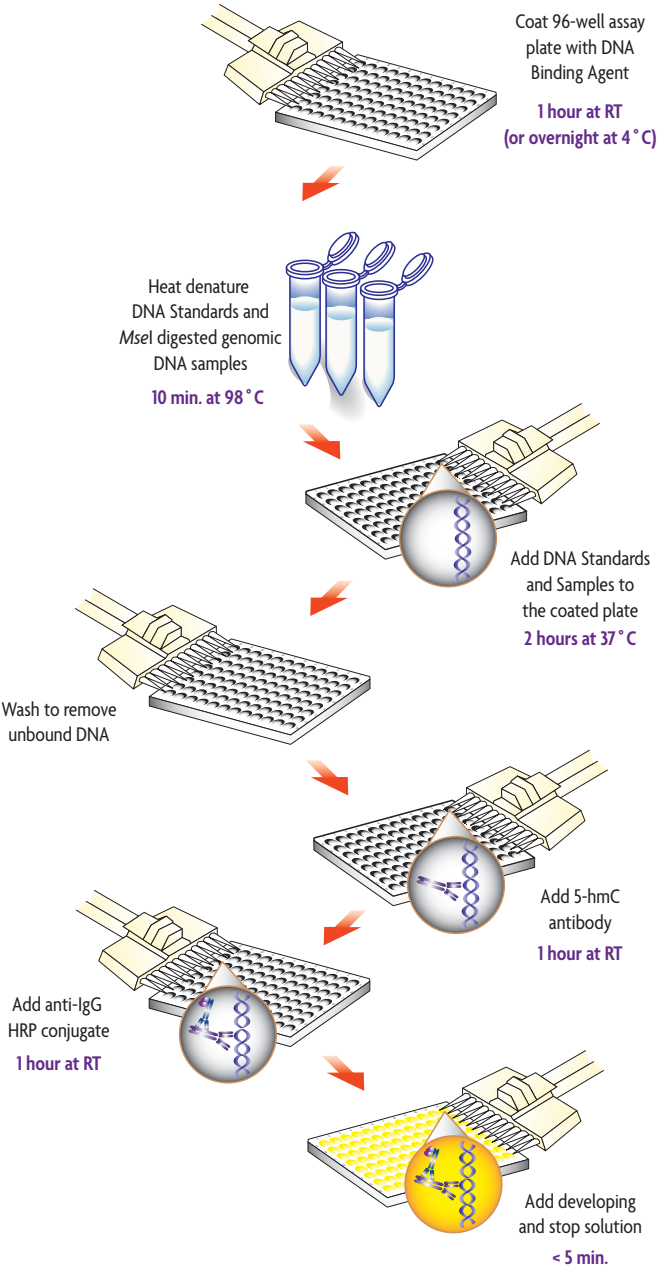
The Global 5-hmC Quantification Kit is designed to detect and quantify 5-hydroxymethylcytosine (5-hmC) in DNA fragments. 5-Hydroxymethylcytosine is an oxidative product of 5-methylcytosine (5-mC) that is catalyzed by the ten eleven translocation (TET) family of enzymes. Research has shown that 5-hmC levels are independent of 5-mC levels and both DNA modifications appear to have distinct biological functions. 5-hmC is known to play a role in transcriptional regulation and embryonic development. It may also serve as a prognostic indicator in certain cancers and neurodegenerative disorders.

In the Global 5-hmC Quantification Kit, genomic DNA of interest is fragmented by enzymatic digestion and heat denatured to create ssDNA. 96-stripwell plates are coated with a DNA binding agent to enhance the capture of DNA fragments to the plate. Following the addition of ssDNA to the coated wells, unbound DNA fragments are washed away. A primary antibody specific for 5-hmC and a secondary antibody conjugated to horseradish peroxidase (HRP) are used for detection of hydroxymethylated fragments. The colorimetric readout is easily quantified by spectrophotometry using a microplate reader at 450nm.

Each kit contains an optimized protocol and reagents necessary to perform DNA fragmentation, plate coating, capture, and colorimetric detection of 5-hydroxymethylcytosine. For added convenience, DNA standards containing known levels of 5-hmC are included in the kit. By generating a standard curve, the 5-hmC levels in each DNA sample can be determined.

| product | format | catalog no. |
|---|---|---|
| Global 5-hmC Quantification Kit | 1 x 96 rxns | 55018 |

## Flow Chart of Process



## Introduction

### DNA Modification 5-Hydroxymethylcytosine (5-hmC)

In mammals and other vertebrates, DNA methylation usually occurs at the carbon 5 (C5) position of cytosine (5-mC), mostly within CpG dinucleotides. In 2009, Kriaucionis and Heintz and Tahiliani *et al*. discovered another DNA modification, 5-hydroxymethylcytosine (5-hmC), which was observed to be elevated in neurons and embryonic stem cells[1,2]. The 5-hydroxymethylcytosine modification results from the enzymatic conversion of 5-methylcytosine into 5-hydroxymethylcytosine by the TET family of cytosine oxygenases[2,3]. While the precise function of 5-hmC has yet to be determined, it has been postulated that it could represent a pathway to demethylate DNA, as 5-hydroxymethylcytosine is repaired as mismatched DNA and replaced with unmethylated cytosine[3]. Alternatively, 5-hmC may be produced by the addition of formaldehyde to DNA cytosines by DNMT proteins[4].

DNA base modifications are dynamic marks and their relative concentration at given loci is infludenced by multiple factors including cell and tissue type, the differentiation state of a cell, and the balance between cellular homeostasis and stress response. 5-hmC has been shown to have distinct biological functions and cellular localizations from 5-mC. Proteins that bind specifically to 5-hmC include DNA repair factors, splicing mediators and transcription regulators[5,6]. Experimental evidence indicates that 5-hmC plays a role in transcriptional regulation and embryonic development[7-17].

Loss of global hydroxymethylation is associated with malignant human cancers such as melanoma. Moreover, mutations in TET2 are commonly observed in a wide range of hematopoietic malignancies and are associated with poor patient outcomes[18-20]. Hydroxymethylation is also most abundant in the brain, where it is believed to play an important role in neurological development and memory formation[10, 21-23]. Therefore, 5-hmC may serve as a prognostic indicator in certain cancers and neurodegenerative disorders, such as Alzheimer's disease, Freidrich's ataxia and Huntington's disease.

Using Active Motif's Global 5-hmC Quantification Kit, genomic DNA from different sample types (*e.g*. normal and diseased), treatment conditions, clinical outcomes, or environmental backgrounds can be analyzed for differences in global 5-hydroxymethylcytosine levels. DNA standards are included in the kit to prepare a standard curve for sample quantification. The assay can detect as little as 0.02% 5-hmC. Results obtained with the Global 5-hmC Quantification Kit enable higher throughput processing of samples, require only 10-50 ng of genomic DNA per well and results are consistent with mass spectrometry values for the same DNA samples.

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## Traditional Methods to Study Hydroxymethylcytosine

To date, there are several methods used for Hydroxymethylcytosine analysis:

1. **Mass Spectrometry (LC-MS/MS):** DNA is digested into single nucleosides which are separated according to size. Cytosines, methylated cytosines and hydroxymethylated cytosines are quantified. Variants of this approach include high performance liquid chromatography (HPLC), as well as two dimensional thin layer chromatography and high performance capillary electrophoresis. While these methods are highly quantitative, they require large amounts of DNA and are not easily amenable to high throughput.
2. **Oxidative Bisulfite Sequencing (OxBS-seq):** Bisulfite conversion consists of the treatment of genomic DNA with sodium bisulfite, leading to deamination of unmethylated cytosines into uracil. With OxBS-Seq, an additional oxidative step is used to discriminate between 5-mC and 5-hmC. The oxidation step converts 5-hmC to 5-formylcytosine (5-fC) which is sensitive to deamination by bisulfite. Converted DNA is then PCR amplified and sequenced. Cytosines that remain in the sequence were 5-mC and not 5-hmC. Ox-BS-Seq enables the discrimination of 5-mC and 5-hmC with bisulfite conversion, but is used as a readout for 5-mC. It often requires repeated bisulfite treatments to fully deaminate 5-fC and the oxidation can degrade DNA.
3. **Tet Assisted Bisulfite Sequencing (TAB-Seq):** TAB-Seq utilizes a glucosylation enzyme to protect 5-hmC in genomic DNA. The DNA is then treated with Tet enzyme to convert 5-mC to 5-carboxylcytosine (5-caC). The 5-hmC remains protected from oxidation and will be read as cytosines in the sequencing reaction. While TAB-Seq is ideal for specific quantification of 5-hmC, it relies on the activity of the Tet enzyme which tends to be very inefficient.
4. **Hydroxymethyl Collector-Seq (hMe-Seal):** The Hydroxymethyl Collector-Seq Kit (Active Motif, Cat 55019) utilizes a glucosylation enzyme to add an azide-modified glucose to 5-hmC. A biotin is then attached to the modified glucose and streptavidin beads are used to enrich for DNA fragments containing 5-hmC. This method is highly efficient and specific, but lacks quantification of 5-hmC.
5. **Hydroxymethylated DNA Immunoprecipitation (hMeDIP):** Methylated DNA Immunoprecipitation is an immunocapture technique in which an antibody specific for hydroxymethylcytosine is used to immunoprecipitate genomic DNA fragments containing 5-hmC. Enriched DNA can be used for analysis of the methylation status of a particular gene by PCR, or in combination with NGS. hMeDIP is prone to antibody bias and the technique does not consider total cytosine content.

## Kit Performance and Benefits

The Global 5-hmC Quantification Kit is used to quantify 5-hydroxymethylcytosine levels in genomic DNA when comparing the samples to the provided DNA standards.

**Range of detection:** The dynamic range of the standard curve is 0% to 0.71% 5-hmC. The assay has been shown to detect as little as 0.02% 5-hmC.

Sample DNA can be used in the range of 10 ng - 50 ng per well. If the OD 450nm readings of the samples fall outside the limits of detection for the assay (as determined by the standard curve), we suggest performing a titration of the sample DNA to determine the appropriate quantity to use within the assay. Then perform the rest of the assay at the determined concentration. We recommend a starting concentration of 10 - 25 ng/well for DNA samples containing an elevated concentration of 5-hmC (*e.g.* human brain). We recommend a concentration of 25-50 ng/well for DNA samples containing lower levels of 5-hmC.

**Assay time:** 5 hours

## Global 5-hmC DNA Quantification Kit

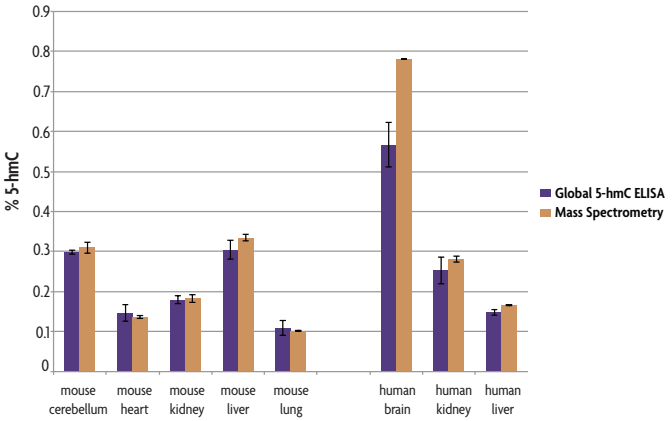


**Figure 1: Comparison of 5-hmC quantification using the Global 5-hmC Quantification Kit and mass spectrometry.**
The Global 5-hmC Quantification Kit was used to determine the % 5-hydroxymethylcytosine in genomic DNA isolated from mouse and human tissues. Genomic DNA was tested in the range of 20 ng/well (human brain) to up to 50 ng/well (all other DNA samples). The % 5-hmC was calculated for each sample using the included DNA Standards. The data was compared to mass spectrometry data obtained from 500 ng of the same DNA samples. Results show the Global 5-hmC Quantification ELISA provides equivalent quantification, while requiring only a fraction of the starting material.

## Global 5-hmC Quantification Kit Components and Storage

Global 5-hmC Quantification Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

| Reagents | Quantity | Storage |
|---|---|---|
| *Mse*I enzyme (10 U/µl) | 20 µl | -20°C |
| 10X *Mse*I Reaction Buffer | 65 µl | -20°C |
| 5-hmC DNA Standards A, B, C, D, E, F & G (10 ng/µl) | 25 µl each (set of 7) | -20°C |
| DNA Binding Agent (lyophilized) | 2 x 60 mg | -20°C |
| 5-Hydroxymethylcytosine pAb | 10 µl | -20°C |
| Anti-rabbit HRP-conj. secondary Ab | 10 µl | 4°C |
| Antibody Dilution Buffer | 12 ml | 4°C |
| 20X Wash Buffer | 25 ml | 4°C |
| Developing Solution | 10 ml | 4°C |
| 96-well plate | 1 ea | 4°C |
| Stop Solution | 10 ml | RT |
| 10X Sample Dilution Buffer | 1 ml | RT |
| 10X Buffer W | 10 ml | RT |
| Plate sealer | 2 ea | RT |
| 0.2 ml PCR stripwell tubes | 1 pk | RT |

**Additional materials required**

- Nanodrop, Qubit or equivalent method to determine DNA concentration
- Incubator set to 37°C
- Thermal cycler or water bath set to 65°C and 98°C
- Ice-water bath
- Orbital shaker (*e.g.* Multi-microplate Genie from Scientific Industries)
- 1.5 or 2 ml microcentrifuge tubes and 15 ml conical tube
- Microcentrifuge with PCR tube and microcentrifuge tube adapters
- Multichannel pipette, 10-200 µl pipette tips and plastic reservoirs
- Vortex
- DNase-free sterile water
- Spectrophotometric microplate reader

## Buffer Preparation and Recommendations

***Mse*I enzyme**

The *Mse*I enzyme is provided ready to use at 10 U/µl. Enough enzyme is provided to perform up to 32 DNA digestions as described in Step A of the protocol. Digested DNA is assayed in duplicate.

**10X *Mse*I Reaction Buffer**

The reaction buffer is provided ready to use at a 10X concentration. Please follow the instructions in the protocol for use.

**DNA Samples**

Following *Mse*I digestion to fragment the DNA samples (as described in Section A), DNA must be quantified for accurate analysis in the assay. It is recommended to use a range of 10-50 ng/well of *Mse*I digested DNA in a final volume of 50 µl per well. If you anticipate your sample to contain higher concentrations of 5-hydroxymethylcytosine, such as brain samples, the lower end of the range can be used. For other sample types, we recommend starting with 50 ng/well. It has been reported that cultured cell lines contain very low levels of 5-hydroxymethylcytosine, therefore, samples from cultured cell lines may not be suitable for use in analysis of 5-hmC levels.

We recommend preparing samples for testing in duplicate. DNA samples should be diluted in Sample Dilution Buffer. It is important that your sample fall within the linear range of the DNA Standards for accurate quantification. It may be necessary to test several DNA concentrations to determine the optimal amount for your sample type. The following table provides recommended volumes for preparing samples in duplicate and includes a slight excess to account for pipetting losses. DNA should be prepared in 0.2ml PCR strip tubes for heat denaturation in a thermal cycler.

| Components | 10 ng/well | 20 ng/well | 25 ng/well | 50 ng/well | Blank |
|---|---|---|---|---|---|
| MseI Digested DNA | 22 ng | 44 ng | 55 ng | 110 ng | – |
| 10X Sample Dilution Buffer | 11 µl | 11 µl | 11 µl | 11 µl | 11 µl |
| Distilled water | Up to 110 µl | Up to 110 µl | Up to 110 µl | Up to 110 µl | 99 µl |

A correction factor will be applied during data analysis to compensate for any concentration differences between the samples and the DNA standards.

**DNA Binding Agent**

The DNA binding agent is provided lyophilized and each vial is sufficient to coat 48-wells of the assay plate. Before opening, quick spin the vial for 1 minute at room temperature to collect contents to the bottom of the tube. Add 1 ml sterile water and vortex until fully dissolved. Transfer the entire contents of the vial to a 15 ml conical tube. Add 5 ml sterile water to the conical tube for a total volume of 6 ml and vortex to mix. Use 100 µl/well to coat the assay plate. If coated plates are not to be used immediately, dried plates can be stored in a foil pouch with a desiccant at 4°C and used within 2-weeks. Discard any unused DNA binding agent solution.

**5-hmC DNA Standards**

DNA Standards are provided a concentration of 10 ng/µl. Standards are provided as dsDNA and must be heat denatured before use in the assay. Each DNA standard contains DNA with a known quantity of 5-hydroxymethylation. For quantification of 5-hmC levels in the samples, the entire range of DNA standards should be used to create a standard curve. Sample results are then compared to the DNA standard curve to extrapolate the % 5-hmC in each sample. Enough DNA is provided to perform two standard curves in duplicate.

To improve accuracy of quantification, we recommend using the DNA standards at a concentration equivalent to the amount of test sample. If working with samples containing a high abundance of 5-hmC (*e.g.* brain), we suggest using DNA standards at a concentration of 20 ng/well. If working with samples containing lower amounts of 5-hmC, we suggest using DNA standards at a concentration of 50 ng/well. A correction factor will be applied during data analysis to compensate for any concentration differences between the samples and the DNA standards.

**1X Buffer W** (See the Quick Chart for Preparing Buffers)

Prepare the amount of 1X Buffer W required for the assay. For every 10 ml of 1X Buffer W required, dilute 1 ml 10X Buffer W with 9 ml sterile water.

**Antibody Dilution Buffer**

The antibody dilution buffer is provided ready to use. Pre-warm to room temperature before use.

**5-Hydroxymethylcytosine pAb**

Prepare a 1:2,000 dilution of the 5-hydroxymethylcytosine polyclonal antibody into Antibody Dilution Buffer by performing a two-step dilution. First, prepare a 1:10 dilution into Antibody Dilution Buffer. Then prepare a 1:200 dilution into Antibody Dilution Buffer for the required amount of antibody needed for the experiment. Use 50 µl per well.

| Components | 1 strip (8 wells) | 4 strips (32 wells) | 6 strips (48 wells) | 12 strips (96 wells) |
|---|---|---|---|---|
| **Initial 1:10 Dilution** | | | | |
| 5-Hydroxymethylcytosine pAb | 2 µl | 2 µl | 2 µl | 4 µl |
| Antibody Dilution Buffer | 18 µl | 18 µl | 18 µl | 36 µl |
| Total Volume | 20 µl | 20 µl | 20 µl | 40 µl |
| **Secondary 1:200 Dilution** | | | | |
| 5-hmC pAb pre-diluted 1:10 | 2.25 µl | 8.5 µl | 13 µl | 26 µl |
| Antibody Dilution Buffer | 448 µl | 1.692 ml | 2.587 ml | 5.174 ml |
| Total Required | 450 µl | 1.7 ml | 2.6 ml | 5.2 ml |

**1X Wash Buffer** (See the Quick Chart for Preparing Buffers)

Prepare the amount of 1X Wash Buffer required for the assay as follows: For every 10 ml of 1X Wash Buffer required, dilute 0.5 ml 20X Wash Buffer with 9.5 ml sterile water. Mix gently to avoid foaming. The 1X Wash Buffer may be stored at 4°C for one week. The Tween 20 contained in the 20X Wash Buffer may form visible aggregates, therefore, homogenize the buffer by incubating at 50°C for 2 minutes and mixing prior to use. Use the 1X Wash Buffer at room temperature.

**Anti-rabbit HRP-conj. secondary antibody** (See the Quick Chart for Preparing Buffers)

Prepare a 1:4,000 dilution of anti-rabbit HRP-conj. secondary antibody into Antibody Dilution Buffer. We recommend performing a two-step dilution by first preparing a 1:10 dilution into Antibody Dilution Buffer. Then prepare a 1:400 dilution into Antibody Dilution Buffer for the required amount of antibody needed for the experiment. Use 50 µl per well.

| Components | 1 strip (8 wells) | 4 strips (32 wells) | 6 strips (48 wells) | 12 strips (96 wells) |
|---|---|---|---|---|
| **Initial 1:10 Dilution** | | | | |
| Anti-rabbit HRP-conjugated antibody | 2 µl | 2 µl | 2 µl | 2 µl |
| Antibody Dilution Buffer | 18 µl | 18 µl | 18 µl | 18 µl |
| Total Volume | 20 µl | 20 µl | 20 µl | 20 µl |
| **Secondary 1:400 Dilution** | | | | |
| Anti-rabbit HRP pre-diluted 1:10 | 1.125 µl | 4.25 µl | 6.5 µl | 13 µl |
| Antibody Dilution Buffer | 449 µl | 1.696 ml | 2.594 ml | 5.187 ml |
| Total Required | 450 µl | 1.7 ml | 2.6 ml | 5.2 ml |

**Developing Solution** (See the Quick Chart for Preparing Buffers)

The Developing Solution should be warmed to room temperature before use. The Developing Solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. However, a blue color present in the Developing Solution indicates that it has been contaminated and must be discarded. Prior to use, place the Developing Solution at room temperature for at least 1 hour. Transfer the amount of Developing Solution required for the assay into a secondary container before aliquoting into the wells. After use, discard remaining Developing Solution.

**Stop Solution** (See the Quick Chart for Preparing Buffers)

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container. After use, discard any remaining Stop Solution.

**WARNING:** The Stop Solution is corrosive. Wear personal protective equipment when handling, *i.e.* safety glasses, gloves and labcoat.

## Quick Chart for Preparing Buffers

| Reagents to prepare | Components | For 1 well | For 1 strip (8 wells) | For 4 strips (32 wells) | For 6 strips (48 wells) | For 12 strips (96 wells) |
|---|---|---|---|---|---|---|
| 1X Buffer W | Distilled water | 900 µl | 8.1 ml | 31.5 ml | 45 ml | 90 ml |
| | 10X Buffer W | 100 µl | 900 µl | 3.5 ml | 5 ml | 10 ml |
| | **TOTAL REQUIRED** | **1 ml** | **9 ml** | **35 ml** | **50 ml** | **100 ml** |
| 1X Wash Buffer | Distilled water | 950 µl | 8.55 ml | 33.25 ml | 47.5 ml | 95 ml |
| | 20X Wash Buffer | 50 µl | 450 µl | 1.75 ml | 2.5 ml | 5 ml |
| | **TOTAL REQUIRED** | **1 ml** | **9 ml** | **35 ml** | **50 ml** | **100 ml** |
| Developing Solution | **TOTAL REQUIRED** | **100 µl** | **900 µl** | **3.5 ml** | **5.4 ml** | **10.8 ml** |
| Stop Solution | **TOTAL REQUIRED** | **100 µl** | **900 µl** | **3.5 ml** | **5.4 ml** | **10.8 ml** |

## Assay Protocol

## Read the entire protocol before use.

Prior to starting the Global 5-hmC Quantification Kit, genomic DNA is fragmented using restriction digestion with the provided *Mse*I enzyme. This enzymatic digestion will create the appropriate size range of DNA fragments for optimal detection in the assay.

## Section A: *Mse*I Digestion of Genomic DNA

We recommend preparing high-quality genomic DNA using a commercially available kit or a standard established protocol and determining DNA concentration by UV spectrophotometry or fluorescence-based quantitation. The Global 5-hmC Quantification assay can be performed on approximately 10-50 ng *Mse*I fragmented genomic DNA per well. We suggest using 0.5 µg genomic DNA per sample type for digestion and then performing the assay in duplicate.

1. In microcentrifuge tubes, set up a restriction digest for each DNA sample to be tested. Also, prepare a mock digestion reaction that will be used as a blank for DNA quantification following the digestion reaction. A small loss of DNA is anticipated as part of the digestion process, therefore quantification MUST be performed following *Mse*I digestion to accurately determine the DNA quantity for use in the assay. A single mock digestion reaction can be used as a blank for all the DNA digestions performed on the same day.

| **Reagents** | **DNA Sample** | **Mock Digestion** |
|---|---|---|
| Genomic DNA (0.5 µg) | _____ µl | 0 µl |
| 10X Reaction Buffer | 2 µl | 2 µl |
| *Mse*I enzyme (10 U/µl) | 0.5 µl | 0.5 µl |
| Sterile water | Up to 20 µl | 17.5 µl |
| **Total Volume** | **20 µl** | **20 µl** |

2. Mix well by pipetting and incubate at 37°C for 2-4 hours.
3. Heat-inactivate *Mse*I by incubating the reaction mixture at 65°C for 20 minutes. Quick spin tubes to collect any condensate.
4. Measure the DNA concentration of each sample using the mock digestion as a blank. The Global 5-hmC Quantification assay is very sensitive to DNA concentration so DNA MUST be quantified following *Mse*I digestion. Failure to properly quantify the DNA following digestion will lead to variability in the assay results. Make sure your samples fall within the range of detection for your spectrophotometer or use a more sensitive fluorescence-based quantitation.
5. If the assay will not be performed immediately, the digested DNA can be stored at -20°C.

## Section B: Plate Set-Up

Determine the appropriate number of microwell strips required for testing samples, standards and blank wells in duplicate. Remove the unused strips from the strip holder and place in the aluminum pouch at 4°C. Allow all microwell strips to be used in the assay to warm to room temperature. If less than 8 wells in a strip need to be used, cover the unused wells with a portion of the adhesive plate sealer while you perform the assay. The unused wells are stable at room temperature for the duration of the assay if kept dry. Once the assay is finished, unused wells should be returned to the aluminum pouch and stored at 4°C.

To improve the binding efficiency of the genomic DNA to the assay plate, a DNA Binding Agent is applied to each well. The DNA Binding Agent is provided lyophilized and each vial is sufficient to coat 48-wells of the assay plate. If not used immediately, coated plates should be dried and stored in a foil pouch with a desiccant at 4°C and used within 2-weeks.

Please plan your experimental design and plate coating accordingly. If only a portion of the plate will be coated and used in the assay, be sure to designate whether the stored strips are coated or not. An example plate layout is shown below.

| | DNA Standards | | *Mse*I-digested Samples | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| A | STD A | STD A | Sample 1 | Sample 1 | Sample 9 | Sample 9 |
| B | STD B | STD B | Sample 2 | Sample 2 | Sample 10 | Sample 10 |
| C | STD C | STD C | Sample 3 | Sample 3 | Sample 11 | Sample 11 |
| D | STD D | STD D | Sample 4 | Sample 4 | Sample 12 | Sample 12 |
| E | STD E | STD E | Sample 5 | Sample 5 | Sample 13 | Sample 13 |
| F | STD F | STD F | Sample 6 | Sample 6 | Sample 14 | Sample 14 |
| G | STD G | STD G | Sample 7 | Sample 7 | Sample 15 | Sample 15 |
| H | Blank | Blank | Sample 8 | Sample 8 | Sample 16 | Sample 16 |

## Section C: Plate Coating

Coat the appropriate number of wells as determined in the plate set-up. Each DNA Binding Agent vial is sufficient to coat 48 wells. Coated plates can stored at 4°C and used within 2-weeks.

1. Allow microwell strips to warm to room temperature.
2. Reconstitute the DNA Binding Agent in water and prepare 1X Buffer W as described in the section Buffer Preparation and Recommendations.
3. Add 100 µl of the reconstituted DNA Binding Agent to each well. Cover the wells with the adhesive plate sealer. Incubate at room temperature for 1 hour. (Or overnight at 4°C.)

4. Remove the plate sealer and discard the DNA Binding Agent from the wells of the plate by inverting the plate over a liquid waste receptacle. Then, tap the inverted plate 3 times on absorbent paper towels.
5. Add 150 µl 1X Buffer W to each well and immediately discard by inverting the plate over a liquid waste receptacle. Then, tap the inverted plate 3 times on absorbent paper towels.
6. Repeat the wash 2 more times for a total of three washes.
7. Tap the inverted plate 3-5 times on absorbent paper towels to remove all the liquid. Failure to remove all the buffer at this step will lead to inaccurate 5-hmC quantification. Plate can be allowed to air dry for up to 2 hours. If the strips will not be used immediately, we recommend letting them air dry for 4 hours before storing in the foil pouch with a dessicant.
8. While the plate is drying, prepare the DNA Standards and Samples as described in Section D & Section E.

## Section D: Standard Curve Preparation

The provided 5-hmC DNA Standards can be used to create a standard curve in duplicate for quantification of percent hydroxymethylcytosine in each sample. Use the entire range of standards as shown in the table below. DNA standards should be prepared at a concentration equivalent to the amount of sample to be tested. For optimal accuracy in quantification, we suggest using DNA standards at 20 ng/well for samples with a high abundance of 5-hmC and we suggest using DNA standards at 50 ng/well for samples with a low abundance of 5-hmC. A correction factor will be applied during data analysis to account for any differences between the quantity of the samples and the DNA standards.

1. Prepare a labeled 0.2 ml stripwell PCR tubes for heat denaturation of the DNA standards. Add the appropriate volume of DNA standard and sterile water to each tube to achieve the desired standard curve concentration as described in the chart below.

| | | 20 ng/well | | 50 ng/well | |
|---|---|---|---|---|---|
| | % 5-hmC | Volume of DNA Standard | Volume of Water | Volume of DNA Standard | Volume of Water |
| STD A | 0.71% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |
| STD B | 0.36% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |
| STD C | 0.18% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |
| STD D | 0.09% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |
| STD E | 0.045% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |
| STD F | 0.022% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |
| STD G | 0% | 4.4 µl | 105.6 µl | 11 µl | 99 µl |

2. Vortex to mix thoroughly. Quick spin to collect liquid.
3. Heat denature samples in a thermal cycler programmed to heat the samples from 25°C to 98°C for 10 minutes. Immediately plunge samples into an ice-water bath for 5 minutes. (Denaturation can be performed in combination with DNA samples prepared in Section E.)

## Section E: DNA Sample Preparation

It is recommended to use a range of 10-50 ng/well of *Mse*I digested DNA in a volume of 50 µl per well. If you anticipate your sample to contain higher concentrations of 5-hydroxymethylcytosine, such as brain samples, the lower end of the range can be used. For other sample types, we recommend starting with 50 ng/well. A correction factor will be applied during data analysis to compensate for any concentration differences between the samples and the DNA standards.

1. Determine the appropriate number of 0.2 ml stripwell PCR tubes required for testing samples and blanks in duplicate. Label each tube.
2. Add DNA to the appropriately labeled PCR tube. (*e.g.* For duplicate reactions, add 110 ng digested DNA diluted in Sample Buffer in a final volume of 110 µl.)
3. Prepare blank wells as described in the DNA Sample Preparation Table on page 8.
4. Cap tubes and briefly vortex to mix. Collect liquid to bottom of tubes.
5. Place samples in a thermal cycler and incubate at 98°C for 10 minutes. Immediately plunge samples into an ice-water bath for 5 minutes.

## Section F: Binding to the Coated Plate

1. Quick spin the heat denatured DNA standards and samples to collect any condensate.
2. Add 50 µl of each DNA standard, sample and blank to the coated assay wells according to the plate layout. Ensure the bottom of the wells are completely covered.
3. Cover the plate with adhesive plate sealer and incubate at 37°C for 2 hours.
4. During this incubation, prepare the 5-Hydroxymethylcytosine antibody dilution as described in the section Buffer Preparation and Recommendations.
5. Remove the plate sealer and discard unbound DNA by quickly inverting the plate over a liquid waste receptacle. Then, tap the inverted plate 3 times on absorbent paper towels.
6. Add 150 µl Buffer W to each well and immediately discard as described above.
7. Repeat the wash 2 more times for a total of three washes.

## Section G: Binding of Primary Antibody

1. Add 50 µl of diluted 5-Hydroxymethylcytosine antibody to each well being used, including blank wells. Cover the plate with plate sealer and incubate for 1 hour at room temperature with mild agitation (200-300 rpm on an orbital shaker).

2. During this incubation, prepare the 1X Wash Buffer and the anti-rabbit HRP antibody dilution as described in the section Buffer Preparation and Recommendations.
3. After the incubation, rinse the wells 3 times with 150 µl 1X Wash Buffer.

## Section H: Binding of Secondary Antibody

1. Add 50 µl of diluted HRP-conjugated anti-rabbit antibody to each well being used, including blank wells. Cover the plate with plate sealer and incubate for 1 hour at room temperature with mild agitation (200-300 rpm on an orbital shaker).
2. During this incubation, place the Developing and Stop Solutions at room temperature.
3. After the incubation, rinse the wells 3 times with 150 µl of 1X Wash Buffer.

## Section I: Colorimetric Reaction

1. Remove as much of the final wash as possible by blotting the plate on paper towels.
2. Add 100 µl of room temperature Developing Solution to all wells being used.
3. Incubate from 1 to 3 minutes at room temperature protected from direct light. Please read the Certificate of Analysis supplied with this kit for optimal development time associated with this lot number. Monitor the blue color development in the standard curve wells containing the highest concentrations of hydroxymethylated DNA (STD A) until they turn light to medium blue. The Blank wells should remain clear. Do not overdevelop.
4. Add 100 µl of Stop Solution to all wells. In presence of the acid, the blue color turns yellow.
5. Read absorbance on a spectrophotometer within 5 minutes at 450nm with an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

   Reading the reference wavelength is optional. Most microtiter plate readers are equipped to perform dual wavelength analysis and with the appropriate software, will automatically subtract the reference wavelength absorbance from the test wavelength absorbance. If your plate reader does not have this capability, you may read the plate twice, once at 450 nm and once at 655 nm then manually subtract the 655 nm OD from the 450 nm OD values.

## Section J: Analysis of Results

1. Average the duplicate readings for all Blank, Standard and Sample wells. Then, subtract the averaged Blank OD 450nm values from the average Standard and the averaged Sample values. These are the Net readings.
2. The Blank wells represent the baseline signal from the assay reagents in the absence of DNA. The STD G (0% 5-hmC) value displays the background signal of the assay in the presence of unmethylated DNA samples, while the STD A represents a sample containing 0.71% 5-hmC.

3. For quantitative analysis, plot the % 5-hmC of the DNA Standards along the X-axis and the Net readings for the DNA Standards along the Y-axis. Draw the best curve fit using a graphing software program (*e.g.* Excel Linear Regression), using only the standards in the linear portion of the curve. Since the OD values will vary with color development time, the best curve fit needs to be determined for each experimental run. Extrapolate the % 5-hmC for each DNA sample from the curve.

4. Apply a correction factor to adjust the values for any samples that were run at a different concentration than the DNA Standards.

$$\textbf{Adjusted \% 5-hmC} = (\text{Calculated \% 5-hmC}) \times \frac{(\text{DNA Standard concentration in ng/well})}{(\text{Sample Quantity in ng/well})}$$

Example calculation:

| | | |
|---|---|---|
| Sample Quantity: | 10 ng/well human brain genomic DNA | |
| Net OD 450nm: | 0.645 | |
| Standard curve: | See Figure 2 below | y=1.5131(x) + 0.0277 |
| Calculated % 5-hmC: | | 0.645 = 1.5131(x) + 0.0277 |
| | | x = 0.408 or 0.408% |

$$\text{Adjusted \% 5-hmC} = (0.408\%) \times \frac{20 \text{ ng/well}}{10 \text{ ng/well}} = \textbf{0.82\% 5-hmC}$$

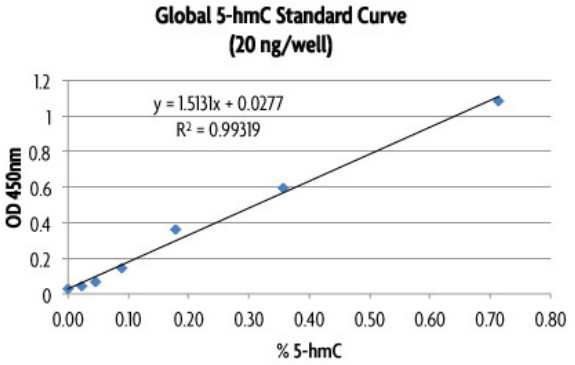


**Figure 2: Graphical representation of the best curve fit as determined by analysis software.**

The % 5 hydroxymethylcytosine represented by each DNA standard was plotted on the x-axis and the Net OD 450nm readings for the DNA standards were plotted on the y-axis. The best curve fit was determined using Excel Linear Regression. The calculated % 5-hmC values for the DNA samples were extrapolated from the curve using the trendline equation.

# Appendix

## Section K: Troubleshooting Guide

| Problem/question | Possible cause | Recommendation |
|---|---|---|
| No signal or weak signal in all wells | Omission of key reagent | Check that all reagents have been added in all wells in the correct order |
| | Substrate or conjugate is no longer active | Test conjugate and substrate for activity by mixing a small aliquot of HRP and Developing Solution together |
| | Enzyme inhibitor present | Sodium azide will inhibit the peroxidase reaction. Follow our recommendations to prepare buffers |
| | Plate reader settings not optimal | Verify the wavelength and filter settings in the plate reader |
| | Incorrect assay temperature | Bring Developing Solution and Stop Solution to room temperature before using |
| | Inadequate volume of Developing Solution | Check to make sure that correct volume is delivered by pipette |
| High background in all wells | Developing time too long | Stop enzymatic reaction as soon as the positive wells turn medium-dark blue or the blank wells turn a faint blue color |
| | Concentration of antibodies is too high | Increase antibody dilutions |
| | Inadequate washing | Ensure all wells are filled with Wash Buffer and follow washing recommendations. |
| Uneven color development between replicates | Incomplete washing of wells | Ensure all wells are filled with Wash Buffer and follow washing recommendations. Ensure no residual wash buffer remains prior to addition of developing solution |
| | Well cross-contamination | Follow washing recommendations |
| | Inaccurate pipetting | Verify that the same amount of sample, primary antibody, secondary antibody, developing and stop solutions are added to each well |
| Signal in sample well beyond the detectable range for the microplate reader | Too much sample per well | Decrease amount of sample per well. Titrate the amount of genomic DNA to use per well such that it falls within the range of the DNA standards. We suggest a range of 10-50 ng/well |
| | Concentration of antibodies is too high | Perform antibody titration to determine optimal working concentration. Start using 1:4000 for primary antibody and 1:5000 for the secondary antibody. The sensitivity of the assay will be decreased |
| No signal or weak signal in sample wells | Not enough sample per well | Increase amount of sample per well. Titrate the amount of genomic DNA to use per well such that it falls within the range of the DNA standards. We suggest a range of 10-50 ng/well |
| No signal or weak signal in standard curve wells | Omission of key reagent | Ensure that all reagents have been added to the wells in the correct order. Verify that DNA standards were prepared as instructed in Section D of the manual. |

## Section L: Related Products

| DNA Methylation | Format | Catalog No. |
|---|---|---|
| MethylDetector™ | 50 rxns | 55001 |
| MethylCollector™ Ultra | 30 rxns | 55005 |
| HypoMethylCollector™ | 30 rxns | 55004 |
| Hydroxymethyl Collector | 25 rxns | 55013 |
| MeDIP | 10 rxns | 55009 |
| hMeDIP | 10 rxns | 55010 |
| DNMT Activity / Inhibition Assay | 1 x 96 rxns | 55006 |
| Methylated DNA Standard Kit | 3 x 2.5 µg | 55008 |
| 5-Carboxylcytosine DNA Standard Kit | 0.5 µg | 55014 |
| Fully Methylated Jurkat DNA | 10 µg | 55003 |
| Recombinant Tet1 protein | 25 µg | 31363 |
| β-Glucosyltransferase enzyme | 500 Units | 55012 |
| PvuRts1I restriction enzyme | 50 Units | 55011 |

| Whole Genome Amplification | Format | Catalog No. |
|---|---|---|
| GenoMatrix™ Whole Genome Amplification Kit | 50 rxns | 58001 |

| Antibodies | Application | Format | Catalog No. |
|---|---|---|---|
| 3-Methylcytosine (3-mC) pAb | DB | 100 µg | 61111 |
| 5-Carboxylcytosine (5-caC) pAb | DB, IF | 100 µl | 61225 |
| 5-Formylcytosine (5-fC) pAb | DB, IF | 100 µl | 61223 |
| 5-Hydroxymethylcytosine pAb | DB, IF, IHC, MeDIP | 100 µl | 39769 |
| 5-Hydroxymethylcytosine mAb | DB, MeDIP | 100 µg | 39999 |
| 5-Methylcytosine mAb | FACS, IHC, IP, MeDIP | 50 µg | 39649 |
| 5-Methylcytosine pAb | DB, MeDIP | 100 µg | 61255 |
| DNMT1 mouse mAb | ChIP, IHC, IP, WB | 100 µg | 39204 |
| DNMT2 rabbit pAb | WB | 100 µg | 39205 |
| DNMT3A mouse mAb | ChIP, IF, IHC, WB | 100 µg | 39206 |
| DNMT3B mouse mAb | ChIP, IF, IP, WB | 100 µg | 39207 |
| DNMT3L rabbit pAb | WB | 100 µl | 39907 |
| MBD1 mouse mAb | WB | 100 µg | 39215 |
| MBD2 mouse mAb | WB | 100 µg | 40965 |
| MBD3 mouse mAb | WB | 100 µg | 39216 |
| MBD4 mouse mAb | WB | 100 µg | 39217 |
| MeCP2 rabbit pAb | WB | 100 µg | 39218 |

Active Motif also offers a growing list of application validated antibodies, including antibodies for histones and histone modifications, transcription factor antibodies, DNA methylation-related antibodies and ChIP validated antibodies. For a complete list go to **www.activemotif.com/abs**

| Histone Purification | Format | Catalog No. |
|---|---|---|
| Histone Purification Kit | 10 rxns | 40025 |
| Histone Purification Mini Kit | 20 rxns | 40026 |

| Chromatin Assembly | Format | Catalog No. |
|---|---|---|
| Chromatin Assembly Kit | 10 rxns | 53500 |
| HeLa Core Histones | 36 µg | 53501 |

## Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.


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