Global 5-hmC DNA ELISA

(version A1)

Catalog No. 55025

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TABLE OF CONTENTS **Page**

Overview

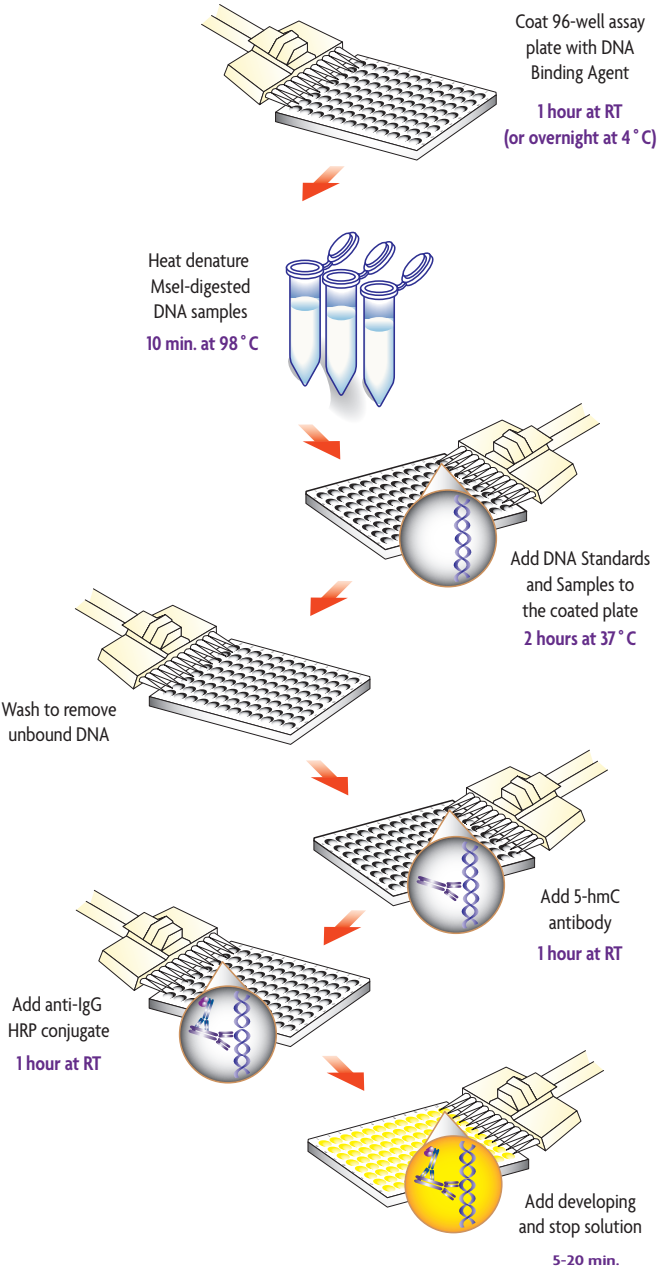
The Global 5-hmC DNA ELISA Kit is designed to detect and quantify 5-hydroxymethylcytosine (5-hmC) in DNA fragments. 5-hmC is an oxidative product of 5-methylcytosine (5-mC) that is catalyzed by the ten eleven translocation (TET) family of enzymes. Research has shown that 5-hmC levels are independent 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 DNA ELISA Kit, genomic DNA of interest is fragmented by enzymatic digestion and heat denatured to create single-stranded DNA (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 bound 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-hmC. For added convenience, a DNA standard containing a known percentage of 5-hmC modification is 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 DNA ELISA Kit	1 x 96 rxns	55025

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-hmC modification results from the enzymatic conversion of 5-mC into 5-hmC 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-hmC 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 influenced 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 DNA ELISA 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-hmC levels. A DNA standard is included in the kit to prepare a standard curve for sample quantification. The assay can detect as little as 0.16% 5-hmC in tissue derived DNA. Results obtained with the Global 5-hmC DNA ELISA Kit enable higher throughput processing of samples, require only 5-10 ng of genomic DNA per well.

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Kit Performance and Benefits

The Global 5-hmC DNA ELISA Kit is used to quantify 5-hmC levels in genomic DNA when comparing the samples to serial dilutions of the provided DNA standard.

Range of detection: The dynamic range of the standard curve is 2.4×10^{-14} to 1.55×10^{-12} moles (0.484 nM – 31 nM) of 5-hmC. The assay has been shown to detect as little as 0.16% 5-hmC in tissue derived genomic DNA.

Sample DNA can be used in the range of 5 ng - 30 ng per well. However, our data indicate that the best agreement between ELISA and mass spectrometry was obtained when 5-10 ng of input DNA was used. For tissue samples containing high levels of 5-hmC (ex. brain) we recommend starting with 5-10 ng/well. For tissue samples containing lower levels of 5-hmc, a concentration of 10-30 ng/well is recommended, with 10 ng providing the best agreement with mass spectrometry. 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.

Assay time: 6 hours

Global 5-hmC DNA ELISA Kit Components and Storage

Global 5-hmC DNA ELISA 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
*Mse*I Reaction Buffer (10X)	65 µl	-20°C
5-hmC DNA Standard (20X, 100 ng/µl)	4 µl	-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	10 ml	4°C
20X Wash Buffer	25 ml	4°C
Developing Solution	11 ml	4°C
Buffer W2 (2X)	70 ml	4°C
96-well plate	1 ea	4°C
Stop Solution	11 ml	RT
Plate sealer	4 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 98°C
- Ice bucket and ice
- Orbital shaker (*e.g.* Multi-microplate Genie from Scientific Industries)
- 1.5 or 2 ml microcentrifuge tubes and 15 ml conical tubes
- Microcentrifuge with PCR tube and microcentrifuge tube adapters
- Multichannel pipette, 10-200 µl pipette tips and plastic reservoirs
- Vortex
- DNase-free sterile water
- Distilled water
- Spectrophotometric microplate reader capable of reading at 450 nm with optional reference wavelength of 655 nm.

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.

10X *Mse*I Reaction Buffer

The reaction buffer is provided at a 10X concentration.

5-hmC DNA Standard

The 5-hmC DNA standard for the Global 5-hmC DNA ELISA Kit is a synthetic oligo containing 9.5% 5-hmC modification, provided at a concentration of 100 ng/µl. A standard curve will be prepared from the oligo stock by performing a seven point 1:2 serial dilution with a top input of 5 ng oligo (7.74×10^{-13} moles oligo, 1.55×10^{-12} moles 5-hmC). Sample results are then compared to the standard curve to extrapolate number of moles 5-hmC, % 5-hmC or molarity of 5-hmC in each sample.

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 1X Buffer W2 and vortex until fully dissolved. Transfer the entire contents of the vial to a 15 ml conical tube. Add 5 ml 1X Buffer W2 to the conical tube for a total volume of 6 ml and vortex to mix.

Antibody Dilution Buffer

The Antibody Dilution Buffer is provided ready to use. IMPORTANT: Bring to room temperature before use.

1X Buffer W2 (See the Quick Chart for Preparing Buffers)

For every 10 ml of 1X Buffer W2 required, combine 5 ml 2X Buffer W2 with 5 ml distilled water. Each half plate (48 wells) requires 43 ml of 1X Buffer W2. We recommend preparing 60 ml. Use the 1X Buffer W2 at room temperature.

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

For every 10 ml of 1X Wash Buffer required, combine 0.5 ml 20X Wash Buffer with 9.5 ml distilled water. Each half plate (48 wells) will require 43 ml of 1X Wash Buffer. We recommend preparing 60 ml. 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.

Developing Solution (See the Quick Chart for Preparing Buffers)

The bottle of Developing Solution should be placed at room temperature for 1 hour before use. Transfer the amount of Developing Solution required for the assays into a secondary container before aliquoting into the wells. After use, discard remaining Developing Solution.

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. However, this does not affect product performance. A blue color in the Developing Solution indicates that it has been contaminated and must be discarded.

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

	Components	6 strips (48 wells)	12 strips (96 wells)
1X Bufer W2	Distilled water	30 ml	60 ml
	2X Buffer W2	30 ml	60 ml
	Total Volume	60 ml	120 ml
1X Wash Buffer	Distilled water	57 ml	114 ml
	20X Wash Buffer	3 ml	6 ml
	Total Volume	60 ml	120 ml
Developing Solution	Total Volume	5.4 ml	10.8 ml
Stop Solution	Total Volume	5.4 ml	10.8 ml

Assay Protocol

Read the entire protocol before use.

Prior to starting the Global 5-hmC DNA ELISA 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. The digested DNA can be stored at -20°C and the assay performed on another day.

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. Determine DNA concentration by UV spectrophotometry or fluorescence-based quantification. The Global 5-hmC DNA ELISA can be performed on approximately 5-30 ng *Mse*I fragmented genomic DNA per well. We suggest starting with 0.5 µg of genomic DNA per sample type for the 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 (vol. will vary)	0 µl
MseI Reaction Buffer (10X)	2 µl	2 µl
MseI enzyme (10 U/µl)	0.5 µl	0.5 µl
DNase-free 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. The Global 5-hmC DNA ELISA Kit is very sensitive to DNA concentration so DNA MUST be quantified following *Mse*I digestion to account for small changes in concentration that can occur as a result of the incubation. Measure the DNA concentration via absorbance spectrophotmetry (ex. NanoDrop™) of each sample using the mock digestion as a blank. Alternatively you can use fluorescence-based quantitation (ex. Qubit™).

Note: If the assay will not be performed immediately, the digested DNA can be stored at -20°C.

Section B: Plate Set-Up

The 96 well plate included in the kit contains 12 sets of 8-well strips. 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 at a time or 6 strips (48 wells). Wells can be coated and used the same day as the assay or allowed to incubate overnight at 4°C with the DNA Binding Agent and used the next day.

Take out the 96 well plate from the foil pouch. Remove six of the 8-well strips from the strip holder and place back into the pouch for long term storage at 4°C and later coating. Allow the strip holder and remaining six strips to warm to room temperature.

Each sample, standard and blank should be conducted in duplicate. Plan your experiment accordingly with two columns for standards and blanks and the remaining four columns for up to 16 samples in duplicate.

An example plate layout is shown below.

	DNA Standards		MseI-digested DNA			
	1	2	3	4	5	6
A	5 ng	5 ng	Sample 1	Sample 1	Sample 9	Sample 9
B	2.5 ng	2.5 ng	Sample 2	Sample 2	Sample 10	Sample 10
C	1.25 ng	1.25 ng	Sample 3	Sample 3	Sample 11	Sample 11
D	0.625 ng	0.625 ng	Sample 4	Sample 4	Sample 12	Sample 12
E	0.3125 ng	0.3125 ng	Sample 5	Sample 5	Sample 13	Sample 13
F	0.156 ng	0.156 ng	Sample 6	Sample 6	Sample 14	Sample 14
G	0.078 ng	0.078 ng	Sample 7	Sample 7	Sample 15	Sample 15
H	Blank	Blank	Sample 8	Sample 8	Sample 16	Sample 16

Section C: Plate Coating

1. Be sure the 6 strips (48 wells) and strip holder have been warmed to room temperature.
2. Prepare 1X Buffer W2 according to the Quick Chart for Preparing Buffers, which will be enough for the rest of the protocol.
3. Before opening, quick spin the vial of DNA Binding Agent for 1 minute at room temperature to collect contents to the bottom of the tube. Add 1 ml 1X Buffer W2 and vortex until fully dissolved. Transfer the entire contents of the vial to a 15 ml conical tube. Add 5 ml 1X Buffer W2 to the conical tube and vortex to mix.
4. 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.

5. 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 on absorbent paper towels untill the excess liquid is removed from the wells.
6. To wash the wells add 150 µl 1X Buffer W2 to each well and immediately discard by inverting the plate over a liquid waste receptacle. Then tap the inverted plate on absorbent paper towels untill the excess liquid is removed from the wells.
7. Repeat the wash 2 more times for a total of three washes.
8. Tap the inverted plate 3-5 times on absorbent paper towels to remove all the liquid. The plate can be used immediately or allowed to air dry inverted for up to 4 hours. If it cannot be used the same day seal and place at 4°C overnight for use the next day.

Section D: Standard Curve Preparation

A standard curve will be created by making serial dilutions of the 5-hmC DNA standard. Enough dilution will be made for each in duplicate.

1. In a new microcentrifuge tube, create a 5 ng/µl working stock of the 5-hmC DNA Standard by performing a 1:20 dilution, combining 2 µl 5-hmC DNA Standard (20X, 100 ng/µl) with 38 µl Buffer W2. Pipet up and down 5-7 times to mix.
3. Take out one strip of the 0.2 ml PCR Stripwell tubes. Pipet 245 µl Buffer W2 into the first tube of the strip. Then pipet 110 µl Buffer W2 into the seven remaining tubes.
4. To perform the standard curve serial dilution, transfer 5 µl of the 5 ng/µl working stock to the first tube of the strip (contains 245 µl Buffer W2) to make a concentration of 0.1 ng/ul. Pipet up and down 5-7 times to mix contents, then transfer 110 µl of this mixture to the next tube. Pipet up and down 5-7 times to mix contents. Then transfer 110 µl to next well. Repeat in this manner through the seventh tube. The last tube (tube 8) is buffer only and will serve as the assay blank.

Section E: DNA Sample Preparation

First be sure that the DNA concentration of each MseI digested sample was quantified as described at the end of Section A.

Digested DNA samples will be diluted with 1X Buffer W2 for a final concentration of 5-30 ng/well with a final volume of 50 µl per well. It is important that samples 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 the sample type. For tissue samples containing high levels of 5-hmC (ex. brain) we recommend starting with 5-10 ng/well. For tissue samples containing lower levels of 5-hmC, a concentration of 10-30 ng/well is recommended. Our data indicate that the best agreement between ELISA and mass spectrometry was obtained for all tissue types when 10 ng/well of input DNA was used.

DNA will be prepared in the 0.2 ml PCR stripwell tubes for heat denaturation in a thermal cycler.

1. Label a 0.2 ml stripwell PCR tube for each sample DNA. 16 samples will require four strips (16 tubes).
2. Each sample assay requires 50 µl of sample. We recommend making up a total volume of 110 µl in order to make enough for each sample in duplicate plus extra to account for pipetting loss.

   Example: If the sample assay is to to contain 5 ng/well add 11 ng MseI digested DNA to the 0.2 ml stripwell PCR tube and bring up final volume to 110 µl with Buffer W2. For 10 ng/well, add 22 ng MseI disgested DNA and bring up final volume to 110 µl with Buffer W2. Pipet up and down 5-7 times to mix.
3. Cap tubes and briefly vortex to mix. Collect liquid to the bottom of tubes
4. Place samples in a thermal cycler and incubate at 98° C for 10 minutes to denature DNA.
5. Immediately plunge samples into an ice-water bath for at least 5 minutes. Keep samples on ice until ready to add single stranded DNA to the coated plate in Section F.

Section F: Binding DNA to the Coated Plate

1. Quick spin the heat denatured samples to collect any condensate.
2. Add 50 µl of the standards, blanks, and denatured DNA samples in duplicate 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 pAb dilution as described below. Be sure the Antibody Dilution Buffer is brought to room temperature before use.

Prepare a 1:2,000 dilution of the 5-hydroxymethylcytosine pAb 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 the Antibody Dilution Buffer.

Preparing 5-Hydroxymethylcytosine pAb

	Components	6 strips (48 wells)
Initial 1:10 Dilution	5-hmC pAb	2 µl
	Antibody Dilution Buffer	18 µl
	Total Volume	20 µl
Secondary 1:200 Dilution	5-hmC pAb pre-diluted 1:10	13 µl
	Antibody Dilution Buffer	2.587 ml
	Total Volume	2.6 ml

5. Remove the plate sealer and discard liquid by quickly inverting the plate over a liquid waste

receptacle. Then, tap the inverted plate on absorbent paper towels to remove excess.

6. Add 150 µl Buffer W2 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 1:2,000 diluted 5-Hydroxymethylcytosine pAb 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 Anti-rabbit HRP-conj. secondary Ab dilution as described below. Be sure the Antibody Dilution Buffer is brought to room temperature before use.
3. Prepare the 1X Wash Buffer as specified in 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.

Preparing Anti-rabbit HRP-conj. secondary Ab

	Components	6 strips (48 wells)
Initial 1:10 Dilution	Anti-rabbit HRP-conj. secondary Ab	2 µl
	Antibody Dilution Buffer	18 µl
	Total Volume	20 µl
Secondary 1:400 Dilution	Anti-rabbit HRP-conj. pre-diluted 1:10	6.5 µl
	Antibody Dilution Buffer	2.594 ml
	Total Volume	2.6 ml

5. Remove the plate sealer and discard liquid by quickly inverting the plate over a liquid waste receptacle. Then, tap the inverted plate on absorbent paper towels to remove excess.
6. Add 150 µl 1X Wash Buffer to each well and immediately discard as described above.
7. Repeat the wash 2 more times for a total of three washes.

Section H: Binding of Secondary Antibody

1. Add 50 µl of 1:4,000 diluted Anti-rabbit HRP-conj. secondary Ab 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. Remove the plate sealer and discard liquid by quickly inverting the plate over a liquid waste receptacle. Then, tap the inverted plate on absorbent paper towels to remove excess.

4. Add 150 µl 1X Wash Buffer to each well and immediately discard as described above.
5. Repeat the wash 2 more times for a total of three washes.

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. Please read the Certificate of Analysis supplied with this kit for optimal development time associated with this lot. Monitor the blue color development in the standard curve wells so that the color gradient goes from light blue in the lowest concentration well to dark blue in the highest concentration well. The Blank wells should remain clear. Do not overdevelop.

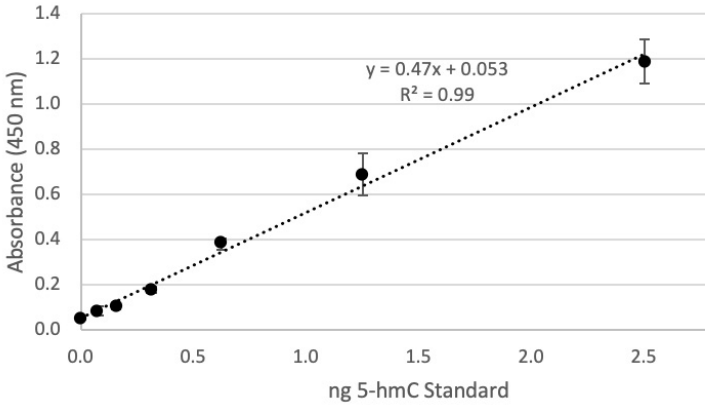
   a. For high abundance samples, 5-7 minute development should be adequate.

   b. For low abundance samples, development time should be 6-8 minutes. However, customers may choose to increase development time to as much as 20-22 minutes to increase signal in the lower range of the standard curve. In this case one or more upper points of the standard curve may need to be discarded.
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 450 nm with an optional reference wavelength of 655 nm. Blank the plate reader according to the manufacturer's instructions using the blank wells.

Section J: Analysis of Results

Generation of Standard Curve

A standard curve can be generated by plotting the raw absorbance values of the 5-hmC Standard serial dilutions. We recommend using curve fitting software to achieve the best fit line. For longer development times, the 5 ng standard may need to be dropped as shown in the example below.



Calculation of % 5-hmC from Raw Absorbance Data

% 5-hmC of samples can be calculated from the standard curve as follows:

First calculate mass of 5-hmC in ng in the sample:

$$\textbf{ng 5-hmC} = \frac{\text{(Sample OD - Y-intercept)}}{\text{(Slope* 9.5}^{\dagger}\text{)}}$$

To obtain % 5-hmC:

$$\textbf{\% 5-hmC} = \frac{\text{(ng 5-hmC)}}{\text{(ng DNA input)}} * 100$$

† 9.5 is a normalization factor to normalize 5-hmC in the standard to 100%, since the oligo contains 9.5% 5-hmC

Calculation of moles of 5-hmC from Raw Absorbance Data

Moles of 5-hmC in samples can be calculated from the standard curve by plotting the standards as moles of 5-hmC against the raw absorbance values. See the table below for the conversion of ng 5-hmC Standard to moles 5-hmC. This assumes a 50 µl volume added per well.

ng 5-hmC	moles 5-hmC	Molarity, 5-hmC (in 50 ul standard volume)
5	1.55E-12	3.10E-08
2.5	7.75E-13	1.55E-08
1.25	3.87E-13	7.75E-09
0.625	1.94E-13	3.87E-09
0.3125	9.68E-14	1.94E-09
0.15625	4.84E-14	9.68E-10
0.078125	2.42E-14	4.84E-10

Downloadable Data Analysis Template

For convenience we have created a data analysis Excel template where you can copy and paste in your raw absorbance values and nanograms of DNA per well and it will calculate both absolute and relative quantification of your unknown samples. It is available for download under the **Documents tab** on the product page.

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 Anti-rabbit HRP-conj. secondary Ab 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 absorbance wavelength (450 nm) of 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 5-30 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 5-30 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.

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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