ELISA PRODUCT INFORMATION & MANUAL

5-Methylcytosine

NBP2-62131

Enzyme-linked Immunosorbent Assay for quantitative detection of Non-species specific 5-Methylcytosine.

For research use only.

Not for diagnostic or therapeutic procedures.

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Novus Biologicals Technical Support if necessary.

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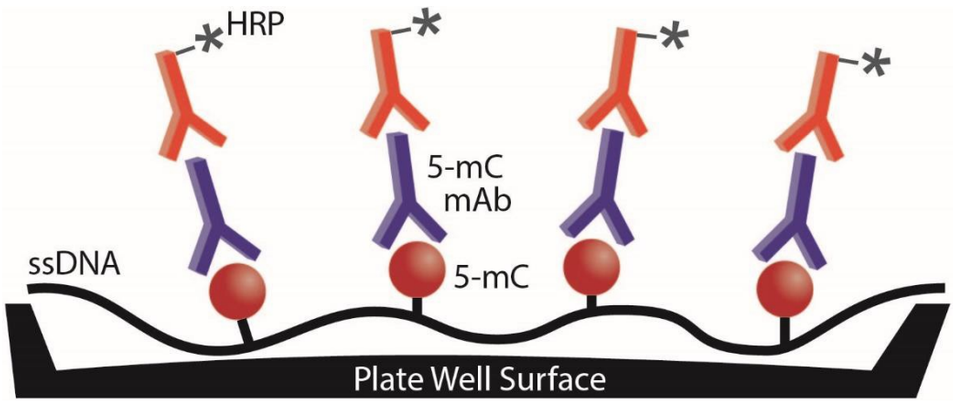
PRODUCT DESCRIPTION

The **5-Methylcytosine DNA ELISA kit** is used to accurately quantify the percent 5-methylcytosine (5-mC) DNA in a species independent manner in a variety of DNA samples including purified genomic DNA and plasmid DNA from microbial, plant and vertebrate sources. PCR amplicons and fragmented (in water, Tris-EDTA or similar) input DNA can also be quantified. This sensitive ELISA kit allows you to obtain results in less than 3 hours that closely correlate to alternative methods like mass spectroscopy (e.g LC-MS/MS-MRM). Sample preparation is minimal and the well-established indirect ELISA protocol is simple and user-friendly. The ELISA kit has been optimized for 100ng input DNA/well and is compatible with DNA in the range of 10-200ng. Detection is less than 0.5% 5-mC per 100ng single-stranded DNA. The 96-well format is ideal for high-throughput global 5-mC DNA detection.



The workflow for the 5-Methylcytosine DNA ELISA kit utilizes the indirect ELISA methodology where denatured, single-stranded DNA (ssDNA) samples are coated on the plate well surfaces and a 5-mC mAb and conjugate HRP-Ab are added to the wells. Indirect detection of 5-mC occurs after addition of the HRP developer.

MATERIALS SUPPLIED

	Size	Storage Temperature
Plate coating buffer	15 mL	4 °C
ELISA Buffer	250 mL	4 °C
5-mC mAb (1 µg/µL)	15 µL	-20 °C
Conjugate HRP-Ab (1µg/µL)	15 µL	-20 °C
HRP developer	15 mL	4 °C
Negative control (100ng/µL)	50 µL	- 20 °C
Positive control (100ng/µL)	50 µL	- 20 °C
1x96-well plate	1 plate	Room Temp.

NOTE: The integrity of kit components is guaranteed for up to six (6) months from date of purchase.

BUFFER PREPARATION AND STORAGE

The coating buffer is stable at room temperature or 4 ºC for extended periods of time**,** and the ELISA buffer should be stored at 4°C and used within 6 months. Alternatively, the ELISA buffer may be dispensed into multiple aliquots and kept at -20°C for long term storage. Avoid repeated freeze/thaw cycles. The HRP developer must be stored at 4 ºC and used within 6 months. Do not freeze. For more rapid color development, bring to room temperature before adding to the wells.

EXPERIMENTAL CONSIDERATIONS

All DNA must be denatured to single-stranded DNA prior to use with the kit. Depending on your experimental design, 10 to 200 ng of sample DNA can be used in the assay despite the optimal detection of 100 ng of single-stranded DNA per well. When using inputs other than 100 ng per well, the amount of control DNA used must be adjusted to equal the amount of sample used. This will ensure accurate % 5-mC quantification. The controls consist of double stranded DNA at a concentration of 100 ng/µL, and can be used for the detection/quantification of 5-mC in DNA. For 5-mC detection, both controls should be assayed. For 5-mC quantification, the negative control should be mixed with the positive control at different ratios to construct a standard curve (see Appendix). The conjugate HRP-Ab is a secondary antibody conjugated to horseradish peroxidase (HRP) and supplied at a concentration of 1 µg/µL.

PROTOCOL

This protocol is optimized for 100 ng of DNA per well, and duplicate samples are recommended for accurate 5-mC detection and quantification. Please read the entire protocol before proceeding.

Preparing the DNA in the plate -- coating steps

1. The wells should be stored in a clean, dry, dark place for later use. Remove the necessary number of well strips to assay DNA samples and controls
2. Add 100 ng of each DNA to a PCR tube and bring the final volume to 100 µL with 5-mC plate coating buffer. For example, if the DNA concentration is 20 ng/µL, add 5 µL of DNA to 95 µL of 5-mC plate coating buffer for a final volume of 100 µL. For more information regarding 5-mC detection and quantification using the negative and positive controls, refer to the Appendix, page 8. Make sure that the volume of the DNA added to the 5-mC plate coating buffer does not exceed 20% of the final volume.
3. Denature the DNA at 98°C for 5 minutes in a thermal cycler.
4. After denaturation, transfer immediately to ice for 10 minutes.
5. Add the denatured DNAs to the wells of the plate.
6. Cover with foil.
7. Incubate at 37 ºC for 1 hour.

Washing and blocking steps

1. Discard the buffer from the wells.
2. Wash each well 3 times with 200 µL of ELISA buffer. Discard the buffer after each wash by tapping out any remaining buffer onto a paper towel after emptying a well.
3. Add 200 µL of the ELISA buffer to each well, and the cover the plate with foil and incubate at 37 ºC for 30 minutes.

Adding the antibodies steps

1. Discard buffer from the wells.
2. Prepare an antibody mix consisting of 5-mC Ab and conjugate HRP-Ab in ELISA buffer according to the following table below. Note the antibody mix can be prepared during the blocking step and kept on ice until it is needed.

	Dilution	Volume (µl)	Example
ELISA buffer	N/A	(# wells + 2) 100	2,000 µL
5-mC Ab	1:2000	Buffer Vol. / 2,000	1 µL
Conjugate HRP-Ab	1:1000	Buffer Vol. / 1,000	2 µL

3. Add 100 µL of this antibody mix to each well. Cover the plate with foil and incubate at 37ºC for 1 hour.

Developing the color and reading steps

1. Discard the antibody mix from the wells. Wash each well 3 times with 200 µL of ELISA Buffer.
2. Add 100 µL of HRP Developer to each well.
3. Allow color to develop for 10-60 minutes at room temperature. The development time will depend on the temperature of the HRP developer.
4. Use an ELISA plate reader to measure the well absorbance at 405-450 nm.

APPENDIX

Analysis with the DNA negative and positive control

The controls should always be included together with the samples for every experiment.

5-mC Detection

The presence or absence of 5-mC can be determined by comparing the absorbance of samples to negative (0% methylation) and positive (100% methylation) controls. Note that the controls must be included on the same plate as the DNA samples for each assay.

5-mC Quantification

To quantify the percentage of 5-mC in a DNA sample, a standard curve must be generated. Note that a new standard curve should be generated for each assay. This is done by preparing mixtures of the negative control (100 ng/µL) and positive control (100 ng/µL) to generate standards of known 5-mC percentage (see table below). The number of standard curve mixtures for 5-mC quantification can vary. In the example given in the table, seven mixtures were prepared. Leftover mixtures can be frozen at or below -20ºC for future use. These must be prepared prior to denaturation and assayed in parallel with the samples. Add 1 µL (i.e., 100 ng) of each mixture to a PCR tube and bring the final volume to 100 µL with 5-mC plate coating buffer. Proceed with the coating steps of the protocol.

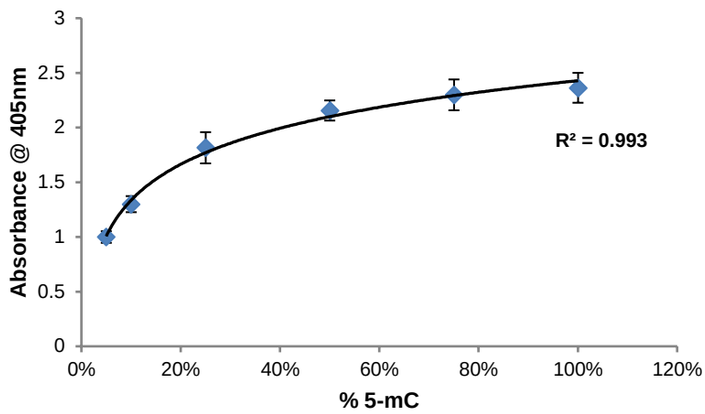
% 5-mC	Negative Control (100 ng/µL)	Positive Control (100 ng/µL)
0%	10.0 µL	0 µL
5%	9.5 µL	0.5 µL
10%	9.0 µL	1.0 µL
25%	7.5 µL	2.5 µL
50%	5.0 µL	5.0 µL
75%	2.5 µL	7.5 µL
100%	0 µL	10.0 µL

Table highlights the preparation of seven mixtures using the controls to be used to generate a standard curve. Total volume of each is 10 µL at a concentration of 100 ng/µL.

The control DNAs consist of *Escherichia coli* genomic DNA. The positive control DNA has been treated with CpG methylase. The density of CpG dinucleotides varies between species and to accurately quantitate the %5-mC simply multiply the calculated %5-mC by the fold difference in CpG density between *E. coli* and the sample species. For example, *E.coli* CpG density/genome length is 0.075 and mouse CpG density/genome length is 0.0081, therefore, the fold difference between *E. coli* and mouse CpG density is 9.22.

The absorbance for each mixture must be plotted as a function of Absorbance @ 405 nm (Y-axis) vs. % 5-mC (X-axis). Using the equation below, derived from the logarithmic second-order regression, determine the 5-mC percentage for DNA samples (unknowns) based on their absorbance.

$$\%\,\mathbf{5-mC} = \mathbf{e}^{\frac{(\mathbf{Absorbance\ -\ y-intercept})}{\mathbf{Slope}}}$$



The curve was using the absorbance values of the DNA mixtures indicated in the table above. A logarithmic relationship was observed with a correlation of 0.99.

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