

**NOTE: Revised Kit Format
and Protocol**

Product Manual

Global DNA Methylation ELISA Kit (5'-methyl-2'- deoxycytidine Quantitation)

Catalog Number

STA-380	96 assays
STA-380-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

Epigenetic changes refer to stable, heritable, and reversible modifications. DNA methylation is one such epigenetic change and has been shown to be associated with almost every biological process. DNA methylation can increase the functional complexity of prokaryotic and eukaryotic genomes by providing additional avenues for the control of cellular processes. DNA methylation is also dynamic and thus can control the timing of cellular events.

In prokaryotes, DNA methylation occurs at the C-5 or N-4 positions of cytosine, as well as the N-6 position of adenine. Multiple enzymes that are capable of methylating bacterial DNA have been identified and are named DNA-MTases. Initially, DNA methylation in bacteria was reported to be associated with restriction-modification systems, wherein foreign DNA that lacks DNA methylation can be recognized and degraded by host methylation-sensitive restriction enzymes. Further studies in bacteria also implicated DNA methylation in the maintenance of the DNA replication fidelity, gene expression regulation, and virulence.

In plants, DNA methylation is found in the sequence context of CpG or CpNpG and has been implicated in normal plant development and regulation of transcription and transposition. At least two classes of DNA methyltransferases (DNMTs) have been identified and characterized in plants. These include the MET1 family of methyltransferases, which preferentially methylate cytosine in CpG context and function both as de novo and maintenance DNMTs, and a second class of DNMTs, named chromomethylases (CMTs), which are unique to plants and methylate cytosine in a CpNpG sequence context.

In mammalian cells, DNA methylation is predominantly found at CpG dinucleotides, most of which reside within gene promoter regions, but in some instances, such as in the case of mouse and human embryonic stem cells, DNA methylation can also be found in non-CpG contexts. DNA methylation in mammalian cells is associated with repression of transcription and maintenance of genomic stability. Due to its important role in genome maintenance and other biological processes, deregulation of DNA methylation is associated with multiple human diseases including cancer. Three major enzymes are required for either de novo methylation (DNMT3A and DNMT3B) or maintenance methylation (DNMT1) in mammalian cells.

There are several methods of detecting total 5-methylcytosine content in the genome. DNA can be digested into single nucleotides and total genomic 5-methylcytosine can be quantitated by high performance liquid chromatography, thin-layer chromatography, or liquid chromatography/mass spectroscopy, but all of these methods are labor intensive and technically demanding.

Cell Biolabs' Global DNA Methylation ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of 5'-methyl-2'-deoxycytidine (5MedCyd) in urine directly, or extracted cell or tissue DNA samples. The quantity of 5MedCyd in an unknown sample is determined by comparing its absorbance with that of a known 5MedCyd standard curve. The kit has a 5MedCyd detection sensitivity range of 300 nM to 40,000 nM. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

Assay Principle

The Global DNA Methylation ELISA Kit is a competitive ELISA for the quantitative measurement of 5MedCyd. The unknown 5MedCyd samples or 5MedCyd standards are first added to the 5MedCyd DNA Conjugate Plate. After a brief incubation, an Anti-5MedCyd monoclonal antibody is added, followed by an HRP conjugated secondary antibody. The 5MedCyd content in unknown samples is determined by comparison with a predetermined 5MedCyd standard curve.

Related Products

1. STA-301: OxiSelect™ BPDE Protein Adduct ELISA Kit
2. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)
3. STA-321: OxiSelect™ DNA Double-Strand Break Assay
4. STA-322: OxiSelect™ UV-Induced DNA Damage ELISA Kit (CPD Quantitation)
5. STA-323: OxiSelect™ UV-Induced DNA Damage ELISA Kit (6-4PP Quantitation)
6. STA-324: OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP Sites)
7. STA-325: OxiSelect™ Oxidative RNA Damage ELISA Kit (8-OHG Quantitation)
8. STA-350: OxiSelect™ Comet Assay Kit (3-Well)
9. STA-355: OxiSelect™ 96-Well Comet Assay Kit
10. STA-357: OxiSelect™ BPDE DNA Adduct ELISA Kit

Kit Components

Box 1 (shipped at room temperature)

1. DNA High-Binding Plate (Part No. 232404): One 96-well strip plate.
2. Anti-5MedCyd Antibody (Part No. 238001): One 30 µL vial.
3. Secondary Antibody, HRP Conjugate (1000X) (Part No. 230003): One 20 µL vial.
4. Assay Diluent (Part No. 310804): One 50 mL bottle.
5. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
6. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
7. Stop Solution (Part. No. 310808): One 12 mL bottle.
8. 5MedCyd Standard (Part No. 238002): One 100 µL vial of 4 mM 5MedCyd in 1X PBS.

Box 2 (shipped on blue ice packs)

1. 100X 5MedCyd DNA Conjugate (Part No. 238005): One 100 µL vial of 100X 5MedCyd DNA Conjugate.

Materials Not Supplied

1. 5MedCyd samples such as urine, or DNA extracted from cells or tissues
2. DNA Extraction Kit
3. Sodium Acetate, pH 5.2
4. Tris Buffer, pH 7.5
5. 1X PBS, 1X PBS containing 0.1% BSA
6. Nuclease P1, Alkaline Phosphatase
7. 10 μ L to 1000 μ L adjustable single channel micropipettes with disposable tips
8. 50 μ L to 300 μ L adjustable multichannel micropipette with disposable tips
9. Multichannel micropipette reservoir
10. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Upon receipt, aliquot and store the 5MedCyd Standard and 100X 5MedCyd DNA Conjugate at -20°C to avoid multiple freeze/thaw cycles. Store all other components at 4°C .

Preparation of Reagents

- 5MedCyd DNA Conjugate Coated Plate: Dilute the proper amount of 100X 5MedCyd DNA Conjugate to 1X in 1X PBS. Add 100 μ L of the 1X 5MedCyd DNA Conjugate to each well and incubate overnight at 4°C . Remove the 5MedCyd DNA Conjugate coating solution and wash once with dH_2O . Blot plate on paper towels to remove excess fluid. Add 200 μ L of Assay Diluent to each well and block for 1 hr at room temperature. Transfer the plate to 4°C and only remove the Assay Diluent immediately before use.

Note: The 5MedCyd DNA Conjugate coated wells are not stable and should be used within 24 hrs after coating. Only coat the number of wells to be used immediately.

- 1X Wash Buffer: Dilute the 10X Wash Buffer to 1X with deionized water. Stir to homogeneity.
- Anti-5MedCyd Antibody and Secondary Antibody: Immediately before use dilute the Anti-5MedCyd Antibody 1:200 and Secondary Antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Preparation of Standard Curve

Thaw the 5MedCyd Standard at room temperature and mix thoroughly by pipetting. Freshly prepare a dilution series of 5MedCyd Standard in the concentration range of 20,000 nM – 313 nM by diluting the 5MedCyd Standard in Assay Diluent (Table 1).

Standard Tubes	4 mM 5MedCyd Standard (μL)	Assay Diluent (μL)	Final 5MedCyd Concentration (μM)
1	5	995	20
2	250 of Tube #1	500	10
3	250 of Tube #2	500	5
4	250 of Tube #3	500	2.5
5	250 of Tube #4	500	1.25
6	250 of Tube #5	500	0.625
7	250 of Tube #6	500	0.313
8	0	500	0

Table 1. Preparation of 5MedCyd Standards

Preparation of Samples

I. Urine

Clear urine samples can be diluted 5- to 50-fold in 1X PBS containing 0.1% BSA before use in the assay. Samples containing precipitates should be centrifuged at 3000 g for 10 minutes, or filtered through 0.45 μm filter, prior to use in the assay.

II. Cell or Tissue DNA Samples

1. Extract DNA from cell or tissue samples by a desired method or commercial DNA Extraction kit.
2. Dissolve extracted DNA in water at 0.1-1 mg/mL.
3. Convert DNA sample to single-stranded DNA by incubating the sample at 95°C for 5 minutes and then rapidly chilling on ice.
4. Digest DNA sample to nucleosides by incubating the denatured DNA with 5-10 units of nuclease P1 for 2 hrs at 37°C in 20 mM Sodium Acetate, pH 5.2, and following with treatment of 5-10 units of alkaline phosphatase for 1 hr at 37°C in 100 mM Tris, pH 7.5.
5. Centrifuge the reaction mixture for 5 minutes at 6000 g and use the supernatant for the 5MedCyd ELISA assay.

Note: Based on LC/MS analysis, there are about 10 ng 5MedCyd per μg of normally methylated human genomic DNA or 1 ng 5MedCyd per μg of hypomethylated human genomic DNA.

Therefore, we recommend using at least 1 μg of digested normally methylated human DNA or 10 μg of digested hypomethylated human DNA per assay.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each 5MedCyd sample including unknown and standard should be assayed in duplicate. High content 5MedCyd samples should be diluted in 1X PBS containing 0.1% BSA.

2. Add 50 μL of unknown sample or 5MedCyd standard to the wells of the 5MedCyd DNA Conjugate Coated Plate. Incubate at room temperature for 10 minutes on an orbital shaker.
3. Add 50 μL of the diluted anti-5MedCyd antibody to each well, mix well and incubate at room temperature for 2 hours on an orbital shaker.
4. Wash microwell strips 4 times with 250 μL 1X Wash Buffer per well with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
5. Add 100 μL of the diluted Secondary Antibody-HRP Conjugate to all wells.
6. Incubate at room temperature for 1 hour on an orbital shaker.
7. Wash microwell strips 4 times according to step 4 above. Proceed immediately to the next step.
8. Warm Substrate Solution to room temperature. Add 100 μL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.
Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.
9. Stop the enzyme reaction by adding 100 μL of Stop Solution into each well, including the blank wells. Results should be read immediately (color will fade over time).
10. Read absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figures demonstrate typical Global DNA Methylation ELISA results. One should use the data below for reference only. This data should not be used to interpret actual results.

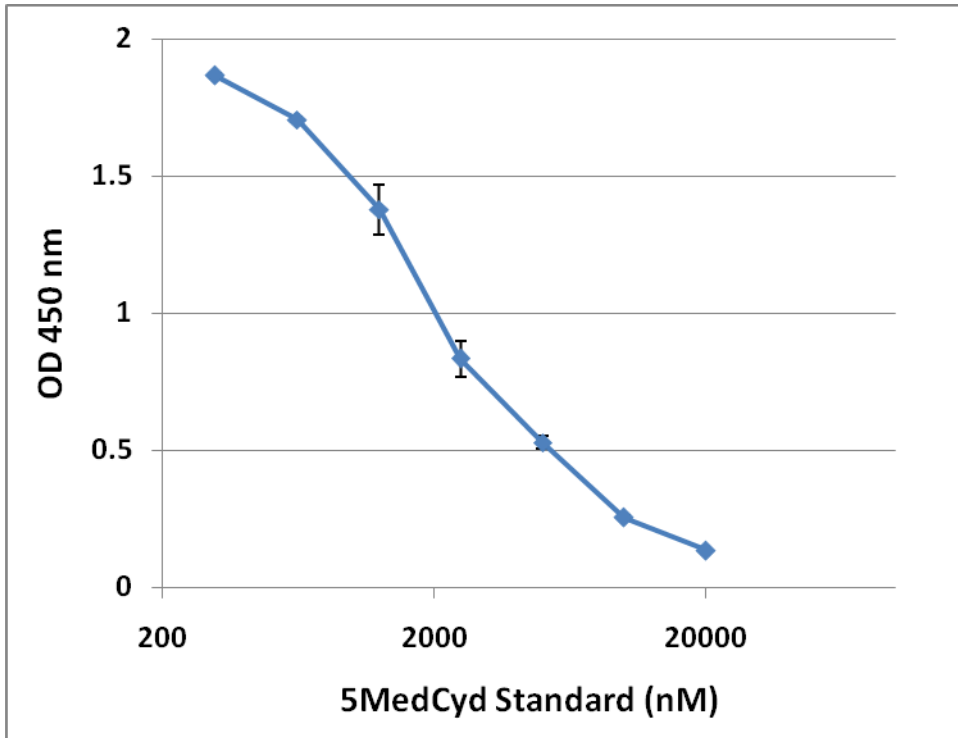


Figure 1: 5MedCyd ELISA Standard Curve.

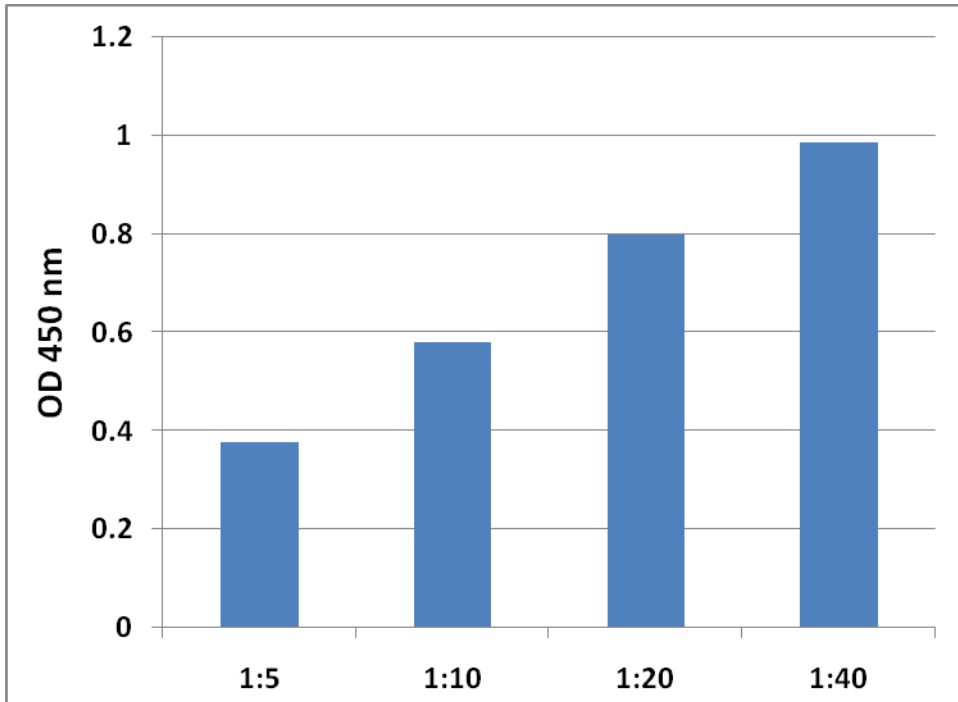


Figure 2: 5MedCyd level in Human Urine Sample. Human urine was first diluted 5-, 10-, 20- and 40-fold in 1X PBS containing 0.1% BSA before use in the assay.

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Recent Product Citations

1. Shah, S.M. et al. (2017). Retinoic acid induces differentiation of buffalo (*Bubalus bubalis*) embryonic stem cells into germ cells. *Gene* doi:10.1016/j.gene.2017.05.037.
2. Lynch, E. W. et al. (2016). Cyclical DNA methyltransferase 3a expression is a seasonal and oestrus timer in reproductive tissues. *Endocrinology*. doi:10.1210/en.2015-1988.
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