Product Manual

5-Carboxylcytosine (5-caC) ELISA Kit

Catalog Number

MET-5103	96 assays
MET-5103-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

DNA methylation is an epigenetic change and has been shown to be associated with almost every biological process. It is essential for normal development and is associated with a variety of important processes such as aging, carcinogenesis, X-chromosome inactivation, repression of transposable elements, and genetic imprinting. Methylation can increase the functional complexity of prokaryotic and eukaryotic genomes by providing additional avenues for the control of cellular processes such as involved in gene expression. DNA methylation is also dynamic and thus can control the timing of cellular events. Methylation has been demonstrated in both cytosine and adenine bases.

Cytosine methylation is the most common DNA methylation which is catalyzed via DNA methyltransferases (DNMT). 5-methylcytosine (5-mC) is a common modified base found in plants and animals and has been termed the "fifth base" in DNA due to its abundance in brain and embryonic stem (ES) cells and role in regulating transcription. In mammals, methylation almost exclusively occurs in CpG nucleotide sites by the addition of methyl group to the fifth carbon of the cytosine ring. S-adenosylmethionines (SAMs) are a common electrophilic donor in the formation of 5-mc. Cytosine oxygenases, such as the ten-eleven translocation (TET) enzymes, can convert 5-methylcytosine to 5-hydroxymethylcytosine (5-hmC). 5-hmc has been called a "sixth" base. Further research of the TET methylation pathway has shown that in addition to 5-hmc, 5-carboxylcytosine (5-caC) and 5-formylcytosine (5-fC) are also formed and both can be cleaved by Thymine DNA glycosylase (TDG). This reaction restores the molecule to a normal cytosine via the base excision repair (BER) process and an active demethylation of 5-mC. Dysregulation of 5-mC pathways has been discovered in various physiological disease states, including cancer.

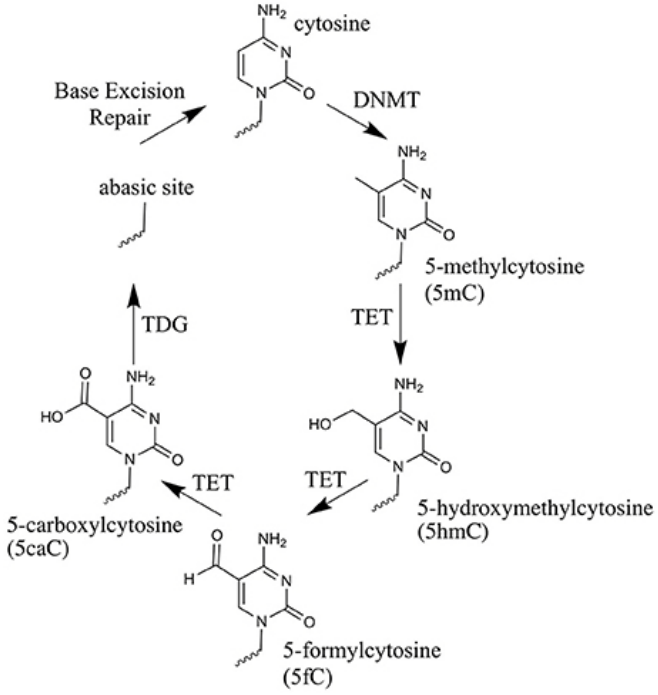

Figure 1: The Cytosine Oxidation Cycle. The cytosine methylation and demethylation pathway. The chemical structures of cytosine, 5-methylcytosine, and each subsequent cytosine modified nucleotide within DNA.

5-carboxylcytosine (5-caC) is a recently discovered modified base in mammalian tissue and cells. It is the final oxidized derivative of 5-mC formed by the oxidation of 5-hmc through TET hydroxylases. Studies have shown that both 5-fC and 5-caC may act as relevant epigenetic markers and as oxidation intermediates. Elevated levels have been confirmed in human breast cancer. Current research is trying to determine if this demethylation model works in vertebrae differentiation and development. The function of 5-fC in gene regulation is not yet entirely clear, but it does appear to have its own functional role in pausing and precision of RNA polymerase II. 5-caC has been detected in mouse embryonic stem cells and in somatic cells of developing follicles. Immunostaining has revealed 5-caC to be localized predominantly in gene-rich euchromatic regions, but not the within the condensed chromatin.

While several chromatography techniques such as HPLC-MS are used for the detection of 5-caC in tissue and cells, these methods require large quantities of DNA, are time consuming, and have low throughput with high costs. Further research is needed to determine 5-caC's role during DNA methylation and their influence on gene expression.

The 5-Carboxylcytosine (5-caC) ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of 5-carboxylcytosine in serum, plasma, cell or tissue samples. The quantity of 5-carboxylcytosine in unknown samples is determined by comparing its absorbance with that of a known 5-carboxylcytosine standard curve. The kit has a 5-carboxylcytosine detection sensitivity of approximately ~50 ng/mL. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

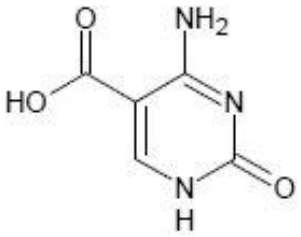

Figure 2: 5-carboxlycytosine (5-caC)

Assay Principle

The 5-Carboxylcytosine (5-caC) ELISA kit is a competitive ELISA for the quantitative measurement 5-carboxylcytosine (5-caC). The unknown 5-carboxylcytosine samples or 5-carboxylcytosine standards are first added to a 5-carboxylcytosine-BSA conjugate preabsorbed microplate. After a brief incubation, an anti-5-carboxylcytosine antibody is added, followed by an HRP conjugated secondary antibody. The 5-carboxylcytosine content in unknown samples is determined by comparison with predetermined 5-carboxylcytosine standard curve.

Related Products

1. MET-5097: N6-Methyladenosine (m6A) ELISA Kit
2. MET-5099: 1-Methyladenosine (m1A) ELISA Kit

3. MET-5102: 5-Formylcytosine (5-fC) ELISA Kit
4. STA-320: OxiSelect™ Oxidative DNA Damage ELISA Kit (8-OHdG Quantitation)
5. STA-321: OxiSelect™ DNA Double-Strand Break (DSB) Staining Kit
6. STA-322: OxiSelect™ UV-induced DNA Damage ELISA Kit (CPD Quantitation)
7. STA-323: OxiSelect™ UV-induced DNA Damage ELISA Kit (6-4PP Quantitation)
8. STA-324: OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP sites)
9. STA-325: OxiSelect™ Oxidative RNA Damage ELISA Kit (8-OHG Quantitation)
10. STA-357: OxiSelect™ BPDE DNA Adduct ELISA Kit
11. STA-380: Global DNA Methylation ELISA Kit (5'-methyl-2'-deoxycytidine Quantitation)
12. STA-671: S-Adenosylhomocysteine (SAH) ELISA Kit
13. STA-672: S-Adenosylmethionine (SAM) ELISA Kit
14. STA-825: OxiSelect™ Nirosative DNA/RNA Damage ELISA Kit (8-Nitroguanine Quantitation)

Kit Components

Box 1 (shipped at room temperature)

1. 96-well Protein Binding Plate (Part No. 231001): One 96-well strip plate.
2. Anti-5-Carboxylcytosine Antibody (Part No. 51031C): One 5 µL vial of anti-5-carboxylcytosine.
3. Secondary Antibody, HRP Conjugate (Part No. 231009): 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.

Box 2 (shipped on blue ice packs)

1. 5-Carboxylcytosine Standard (Part No. 51032D): One 100 µL vial of 1 mg/mL 5-carboxylcytosine-BSA.
2. 5-Carboxylcytosine Conjugate (Part No. 51033D): One 10 µL vial of 5-carboxylcytosine conjugate in PBS.

Materials Not Supplied

1. 5-carboxylcytosine samples such as urine, serum, plasma or DNA extracted from cells or tissues.

2. DNA Extraction Kit
3. Sodium Acetate, pH 5.2
4. Tris Buffer, pH7.5
5. Nuclease P1, Alkaline Phosphatase
6. 10 kDa molecular weight cutoff (MWCO) centrifuge spin filter (e.g. Amicon Ultra 0.5mL)
7. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
8. 50 µL to 300 µL 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 5-Carboxylcytosine Standard and the 5-Carboxylcytosine Conjugate at -80ºC and avoid multiple freeze/thaw cycles. Store the Anti-5-Carboxylcytosine Antibody at -20ºC. Store all remaining kit components at 4ºC.

Preparation of Reagents

- 5-Carboxylcytosine Conjugate Coated Plate: Dilute the proper amount of 5-Carboxylcytosine Conjugate 1:1000 in 1X PBS depending on the number of required assays. (Example: Add 5 µL of 5-Carboxylcytosine Conjugate stock tube to 4.995 mL 1X PBS to coat 48 wells). Add 100 µL of this diluted 5-carboxylcytosine conjugate coating solution to each well and incubate overnight at 4ºC. Remove the 5-carboxylcytosine conjugate coating solution and blot the plate on paper towels to remove excess fluid. Immediately add 200 µL of Assay Diluent to each well and block for 1-2 hr at room temperature. Transfer the plate to 4ºC and remove the Assay Diluent immediately before use.

  Note: The 5-carboxylcytosine-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-5-Carboxylcytosine Antibody and Secondary Antibody, HRP Conjugate (1000X): Immediately before use, dilute the Anti-5-Carboxylcytosine Antibody 1:1000 and Secondary Antibody 1:1000 with Assay Diluent. Do not store diluted solutions.

Preparation of Standard Curve

Prepare fresh standards by diluting the 5-Carboxylcytosine Standard stock from 1 mg/mL (1000 µg/mL) to 50 µg/mL in Assay Diluent for a 1:20 final dilution. (Example: Add 20 µL of 5-Carboxylcytosine Standard stock tube to 380 µL of Assay Diluent). Continue preparing a dilution series of 5-carboxylcytosine standards in the concentration range of 0 µg/mL to 50 µg/mL by diluting the 5-carboxylcytosine standards in Assay Diluent (Table 1).

Standard Tubes	5-Carboxylcytosine Standard (µL)	Assay Diluent (µL)	5-Carboxylcytosine-BSA (µg/mL)
1	20	380	50
2	200 of Tube #1	200	25
3	200 of Tube #2	200	12.5
4	200 of Tube #3	200	6.25
5	200 of Tube #4	200	3.13
6	200 of Tube #5	200	1.56
7	200 of Tube #6	200	0.78
8	200 of Tube #7	200	0.39
9	200 of Tube #8	200	0.20
10	200 of Tube #9	200	0.10
11	0	200	0

Table 1. Preparation of 5-Carboxylcytosine Standards

Preparation of Samples

I. Serum

Avoid hemolyzed and lipemic blood samples. Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Aliquot samples for testing and store at -80ºC. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.

Note: This assay is not compatible with rabbit serum or plasma due to high levels of rabbit IgG that will cross react with the secondary antibody.

II. Plasma

Avoid hemolyzed and lipemic blood samples. Collect blood with heparin or citrate and centrifuge at 2000 x g and 4ºC for 10 minutes. Remove the plasma layer and store on ice. Avoid disturbing the white buffy layer. Aliquot samples for testing and store at -80ºC. Perform dilutions in Assay Diluent or PBS containing 0.1% BSA as necessary.

Note: This assay is not compatible with rabbit serum or plasma due to high levels of rabbit IgG that will cross react with the secondary antibody.

III. Cell or Tissue DNA Samples

1. Purify DNA from cell or tissue samples by a desired method or commercial DNA Extraction kit.
2. Dissolve purified DNA in nuclease free water at 1-5 mg/mL.
3. Convert DNA sample to single-stranded DNA by incubating the sample at 95ºC for 5 minutes and rapidly chilling on ice.

4. Digest DNA sample to nucleosides by incubating the denatured RNA with 5-20 units of nuclease P1 (previously reconstituted in the manufacturer's recommended buffer) for 2 hrs at 37ºC in a final concentration of 20 mM Sodium Acetate, pH 5.2.
5. Add 5-10 units of alkaline phosphatase (previously reconstituted in the manufacturer's recommended buffer) plus sufficient Tris buffer to a final concentration of 100 mM Tris, pH 7.5, and incubate for 1 hr at 37ºC.
6. Centrifuge the reaction mixture for 5 minutes at 6000 x g and collect the supernatant for use in the ELISA.

Assay Protocol

1. Prepare and mix all reagents thoroughly before use. Each 5-carboxylcytosine sample including unknown and standard should be assayed in duplicate.
2. Add 50 µL of unknown sample or 5-Carboxylcytosine Standard to the wells of the 5-Carboxylcytosine Conjugate Coated Plate. Incubate at room temperature for 10 minutes on an orbital shaker.
3. Add 50 µL of the diluted Anti-5-Carboxylcytosine Antibody to each well, incubate at room temperature for 1 hour on an orbital shaker.
4. Wash microwell strips 3 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 3 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 well on a spectrophotometer using 450 nm as the primary wave length.

Example of Results

The following figures demonstrate typical 5-Carboxylcytosine ELISA results. One should use the data below for reference only. This data should not be used to interpret actual results.

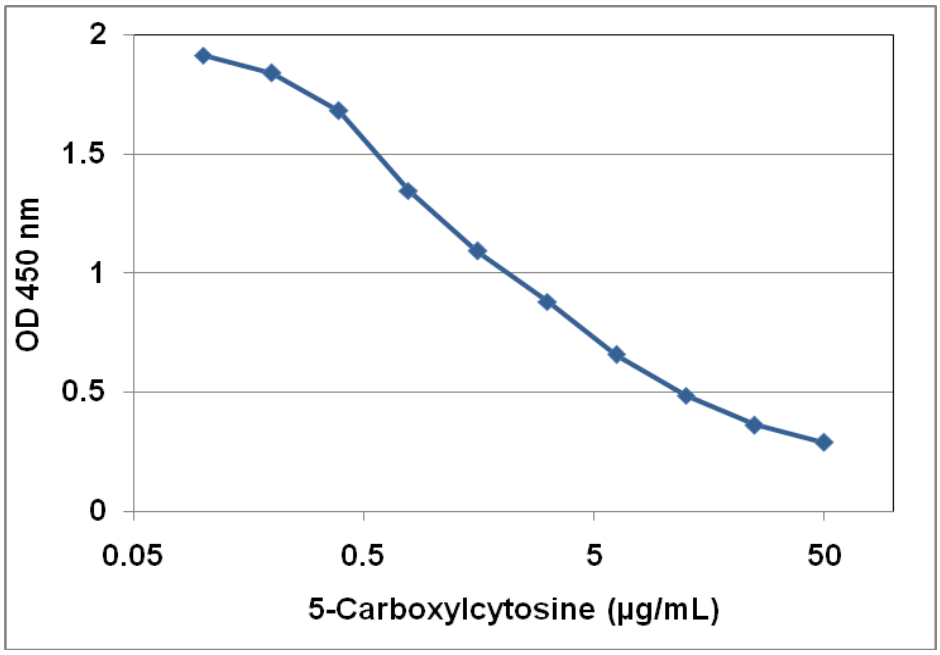

Figure 3: 5-Carboxylcytosine ELISA Standard Curve.

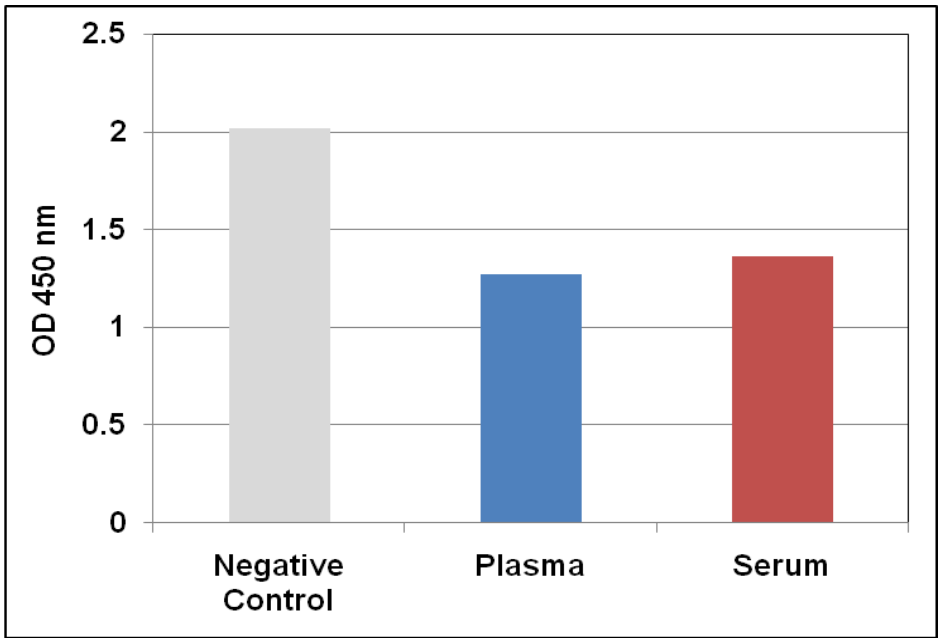

Figure 4: 5-Carboxylcytosine levels in human plasma and serum. Undiluted human samples were tested according to the Assay Protocol instructions.

References

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2. Huang, W., et al. (2016) *Chem. Sci.* **7**: 5495-5502.
3. Ito, S., et al. (2011) *Science* **333(6047)**: 1300-1303.
4. Kellinger, M., et al. (2012) *Nat. Struct. Mol. Biol.* **19(8)**: 831-833.
5. Wu, H., et al. (2011) *Genes Dev.* **25(23)**: 2436-2452.

Warranty

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