Anti-CML mouse autoantibody ELISA Kit
User's Manual
For Research Use Only, Not for use in diagnostic procedures

CircuLex ™

ELISA Kit for Measuring Anti-CML/N$^{\varepsilon}$-(Carboxymethyl)lysine mouse autoantibody

CircuLex Anti-CML mouse autoantibody ELISA Kit

Cat# CY-8067

Intended Use

The CycLex Research Product **CircuLex Anti-CML mouse autoantibody ELISA Kit** is used for the semi-quantitative measurement of IgG class anti-CML/N$^{\varepsilon}$-(Carboxymethyl)lysine mouse autoantibody in mouse serum and plasma.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage
• Upon receipt store all components at 4°C.
• Don't expose reagents to excessive light.

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Introduction

Reducing sugars react with protein amino groups to form a diverse group of protein-bound moieties with fluorescent and cross-linking properties. These compounds, called advanced glycosylation end products (AGEs), have been implicated in the structural and functional alterations of proteins that occur during aging and long-term diabetes.

Although several AGE structures have been reported (1, 2), it was demonstrated that N^ε-(Carboxymethyl)lysine (CML) is a major antigenic AGE structure. CML concentration is also increased in patients who have diabetes with complications, including nephropathy (3–5), retinopathy (6), and atherosclerosis (7–9). CML is also recognized by receptor for AGE (RAGE), and CML-RAGE interaction activates cell signaling pathways such as NF-B and enhances the expression of vascular cell adhesion molecule-1 in human umbilical vein endothelial cells (10).

It has been postulated that AGE structures present in vivo could serve as an immunological epitope to raise autoantibodies against AGE structures, particularly CML. Shibayama et al. showed the presence of autoantibodies against AGE structures, particularly those against CML adduct in streptozotocin (STZ)-induced diabetic rats and patients with several diseases (11, 12). The autoantibody against CML adduct was higher in patients with renal failure than in normal subjects or diabetic patients without renal failure (11). These results suggest that autoantibody against CML might play a possible role in the development of diabetic nephropathy or chronic renal failure.

Principle of the Assay

The CircuLex Anti-CML mouse autoantibody ELISA Kit employs the semi-quantitative enzyme immunoassay technique. CML-BSA or BSA has been pre-coated onto a microplate. Standards or samples are pipetted into the wells. Any anti-CML-adduct autoantibody present is bound by the immobilized CML-BSA but not by the immobilized BSA. After washing away any unbound substances, an HRP conjugated antibody specific for mouse IgG is added to the wells. Following a wash to remove any unbound antibody HRP conjugate, the remaining conjugate is allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured at 450 nm. The absorbance is proportional to the concentration of anti-CML-adduct antibody. A standard curve is constructed by plotting absorbance values versus anti-CML-adduct autoantibody concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

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Summary of Procedure

Add 50 µL of diluted samples or standards to the wells

    ↓   Incubate for 1 hour at room temp.

Wash the wells

↓

Add 50 µL of HRP conjugated anti-mouse IgG antibody

    ↓   Incubate for 1hour at room temp.

Wash the wells

↓

Add 50 µL of Substrate Reagent

↓

Add 50 µL of Stop Solution

↓

Measure absorbance at 450 nm

Materials Provided

All samples and standards should be assayed in duplicate. The following components are supplied and are sufficient for the one 96-well microplate kit.

CML-BSA coated Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with CML-BSA.

BSA coated Microplate: One microplate supplied ready to use, with 96 wells (12 strips of 8-wells) in a foil, zip-lock bag with a desiccant pack. Wells are coated with BSA. Use for reference.

10X Wash Buffer: One 100 mL bottle of 10X buffer containing 2%Tween®-20

Dilution Buffer: One bottle containing 50 mL of 1X buffer; use for sample dilution. Ready to use.

Anti-CML Antibody Standard: One vials containing 64 ng of Anti-CML antibody.

HRP conjugated Detection Antibody: One vial containing 12 mL of HRP (horseradish peroxidase) conjugated anti-mouse IgG antibody. Ready to use.

Substrate Reagent: 20 mL of the chromogenic substrate, tetra-methylbenzidine (TMB). Ready to use.

Stop Solution: One bottle supplied ready to use, containing 20 mL of 1 N H_2SO_4.

Materials Required but not Provided

• **Pipettors:** 2-20 µL, 20-200 µL and 200-1000 µL precision pipettors with disposable tips.

• **Precision repeating pipettor**

• **Orbital microplate shaker**

• **Microcentrifuge and tubes** for sample preparation.

• **Vortex mixer**

• **Microplate washer:** optional (Manual washing is possible but not preferable)

• **Plate reader** capable of measuring absorbance in 96-well plates at dual wavelengths of 450 nm/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. The plate can also be read at a single wavelength of 450 nm, which will give a somewhat higher reading.

• **Software package facilitating data generation and analysis :**optional

• **500 or 1000 mL graduated cylinder**

• **Reagent reservoirs**

• **Deionized water of the highest quality**

• **Disposable paper towels**

Precautions and Recommendations

• Allow all the components to come to room temperature before use.

• All microplate strips that are not immediately required should be returned to the zip-lock pouch, which must be carefully resealed to avoid moisture absorption.

• Do not use kit components beyond the indicated kit expiration date.

• Use only the microtiter wells provided with the kit.

• Rinse all detergent residue from glassware.

• Use deionized water of the highest quality.

• Do not mix reagents from different kits.

• The buffers and reagents used in this kit contain NaN_3 as preservatives. Care should be taken to avoid direct contact with these reagents.

• Do not mouth pipette or ingest any of the reagents.

• Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.

• Dispose of tetra-methylbenzidine (TMB) containing solutions in compliance with local regulations.

• Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide.

• Wear gloves and eye protection when handling immunodiagnostic materials and samples of mouse origin, and these reagents. In case of contact with the Stop Solution and the Substrate Solution, wash skin thoroughly with water and seek medical attention, when necessary.

• **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

• **CAUTION: Sulfuric Acid is a strong acid. Wear disposable gloves and eye protection when handling Stop Solution.**

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Sample Collection and Storage

Serum: Use a serum separator tube and allow samples to clot for 60 ± 30 minutes. Centrifuge the samples at 4°C for 10 minutes at 1,000 x g. Remove serum and assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of serum may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma by use of EDTA-Na$_2$ as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-Na$_2$ and Futhan5 to stabilize the sample against spontaneous *in vitro* complement activation. Immediately centrifuge samples at 4°C for 15 minutes at 1,000 x g. Assay immediately or store samples on ice for up to 6 hours before assaying. Aliquots of plasma may also be stored at below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

   Note: Citrate plasma has not been validated for use in this assay.

Other biological samples: Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles.

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Detailed Protocol

The CycLex Research Product **CircuLex Anti-CML mouse autoantibody ELISA Kit** is provided with removable strips of wells so the assay can be carried out on separate occasions using only the number of strips required for the particular determination. Since experimental conditions may vary, an aliquot of the Anti-CML mouse antibody Standard within the kit, should be included in each assay as a calibrator. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Preparation of Working Solutions

All reagents need to be brought to room temperature prior to the assay. Assay reagents are supplied ready-to-use, with the exception of **10X Wash Buffer** and **Anti-CML Antibody Standard**.

1. Prepare a working solution of Wash Buffer by adding 100 mL of the **10X Wash Buffer** to 900 mL of deionized (distilled) water (ddH$_2$O). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Anti-CML Antibody Standard** with **0.8 mL** of **ddH$_2$O**. The concentration of the anti-CML antibody in vial should be **80 ng/mL**, which is referred as a **Master Standard** of anti-CML antibody.

   Prepare Standard Solutions as follows:
   Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard (Std.1) serves as the highest standard. The **Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Dilution Buffer	Concentration
Std.1	150 µL of Master Standard	450 µL	20 ng/mL
Std.2	300 µL of Std. 1 (20 ng/ml)	300 µL	10 ng/mL
Std.3	300 µL of Std. 2 (10 ng/ml)	300 µL	5 ng/mL
Std.4	300 µL of Std. 3 (5 ng/ml)	300 µL	2.5 ng/mL
Std.5	300 µL of Std. 4 (2.5 ng/ml)	300 µL	1.25 ng/mL
Std.6	300 µL of Std. 5 (1.25 ng/ml)	300 µL	0.63 ng/mL
Std.7	300 µL of Std. 6 (0.63 ng/ml)	300 µL	0.31 ng/mL
Blank	-	300 µL	0 ng/mL

   Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Dilution Buffer before dispensing. Unused portions of Standards should be aliquoted and stored at below -70°C immediately. Avoid multiple freeze and thaw cycles.

Sample Preparation

• Serum and plasma samples require 100-fold dilution.
  e.g. 2 µL sample + 198 µL Dilution Buffer

Assay Procedure

1. Remove the appropriate number of both **CML-BSA coated Microplate** and **BSA coated Microplate** wells from the foil pouch and place them into the well holder. Return any unused wells to the foil pouch, refold, seal with tape and store at 4°C

2. Dilute samples with Dilution Buffer. (See "Sample Preparation" above.)

3. Pipette **50 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells of both **CML-BSA coated Microplate** and **BSA coated Microplate**.

4. Incubate the wells **at room temperature (ca.25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

5. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

6. Add **50 µL** of **HRP conjugated Detection Antibody** into each well.

7. Incubate the plate **at room temperature (ca.25°C) for 1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.

8. Wash 4-times by filling each well with Wash Buffer (350 µL) using a squirt bottle, multi-channel pipette, manifold dispenser or microplate washer.

9. Add **50 µL** of **Substrate Reagent**. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminum foil is recommended. Return Substrate Reagent to 4°C immediately after the necessary volume is removed

10. Incubate the plate **at room temperature (ca. 25°C) for 10-20 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker. The incubation time may be extended up to 30 minutes if the reaction temperature is below than 20°C.

11. Add **50 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

12. Measure absorbance in each well using a spectrophotometric microplate reader at dual wavelengths of 450/540 nm. Dual wavelengths of 450/550 or 450/595 nm can also be used. Read the microplate at 450 nm if only a single wavelength can be used. Wells must be read within 30 minutes of adding the Stop Solution.

   Note-1: Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

   Note-2: Reliable standard curves are obtained when either O.D. values do not exceed 0.35 units for the blank (zero concentration), or 2,0 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

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Calculations

Average the duplicate readings for each standard and sample in CML-BSA coated plate and BSA coated plate. Subtract the average readings in BSA coated plate from those in CML-BSA coated plate. Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation. To determine the anti-CML-adduct concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding anti-CML-adduct concentration. If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

1. The dose-response curve of this assay fits best to a sigmoidal 5-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 5-parameter logistic function. It is important to make an appropriate mathematical adjustment to accommodate for the dilution factor.

2. Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of calibrators versus log of the known concentration (X) of calibrators, using the four-parameter function. Alternatively, the logit log function can be used to linearize the calibration curve (i.e. logit of absorbance (Y) is plotted versus log of the known concentration (X) of calibrators).

Measurement Range

The measurement range is 0.31 ng/mL to 20 ng/mL. Any sample reading higher than the highest standard should be diluted with Dilution Buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the anti-CML mouse autoantibody concentration.

Troubleshooting

1. The anti-CML antibody Standard should be run in duplicate, using the protocol described in the **Detailed Protocol**. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate insufficient washing. If all instructions in the **Detailed Protocol** were followed accurately, such results indicate a need for washer maintenance.

3. Overall low signal may indicate that desiccation of the plate has occurred between the final wash and addition of Substrate Reagent. Do not allow the plate to dry out. Add Substrate Reagent immediately after wash.

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Reagent Stability

All of the reagents included in the CycLex Research Product **CircuLex Anti-CML mouse autoantibody ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, kit reagents should be stored at 4°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

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Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of anti-CML antibody giving absorbance lower than mean absorbance plus three standard deviations of the absorbance of Blank: Blank + 3*SD Blank) is better than 0.162 ng/mL of sample.

Typical Standard Curve
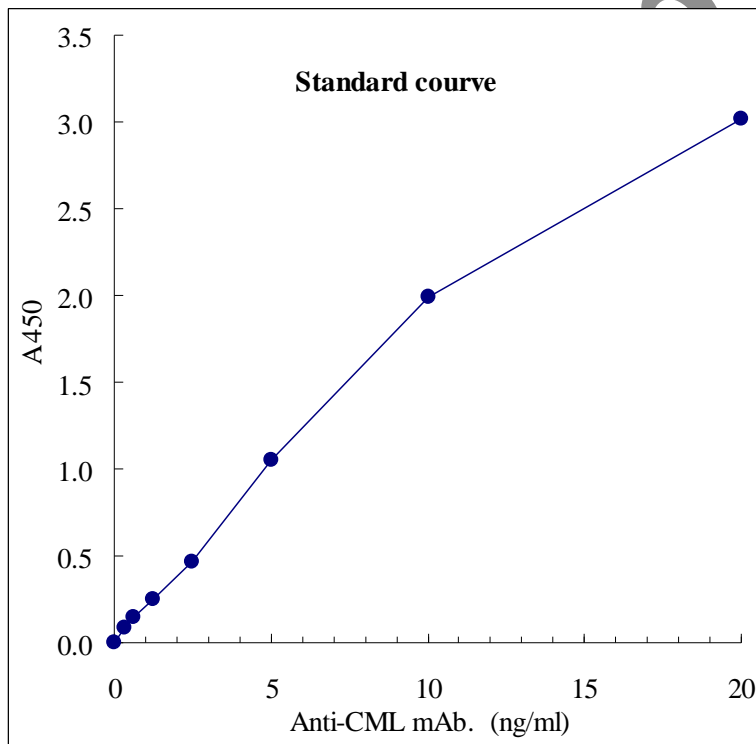
2. Specificity

The antibodies in the CircuLex Anti-CML mouse autoantibody ELISA Kit are highly specific of CML-adduct, with no detectable cross-reactivity to non-CML-protein that may be present in mouse serum/plasma.

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3. Precision

<u>Intra-assay Precision</u> (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=8) CV=5.7 %

<u>Inter-assay Precision</u> (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

• Inter-assay (Run-to-Run, n=4) CV=8.9 %

4. Spiking Recover

Serum samples were spiked with different amounts of anti-CML antibody and assayed.
The recovery of anti-CML antibody spiked to levels throughout the range of the assay was evaluated.
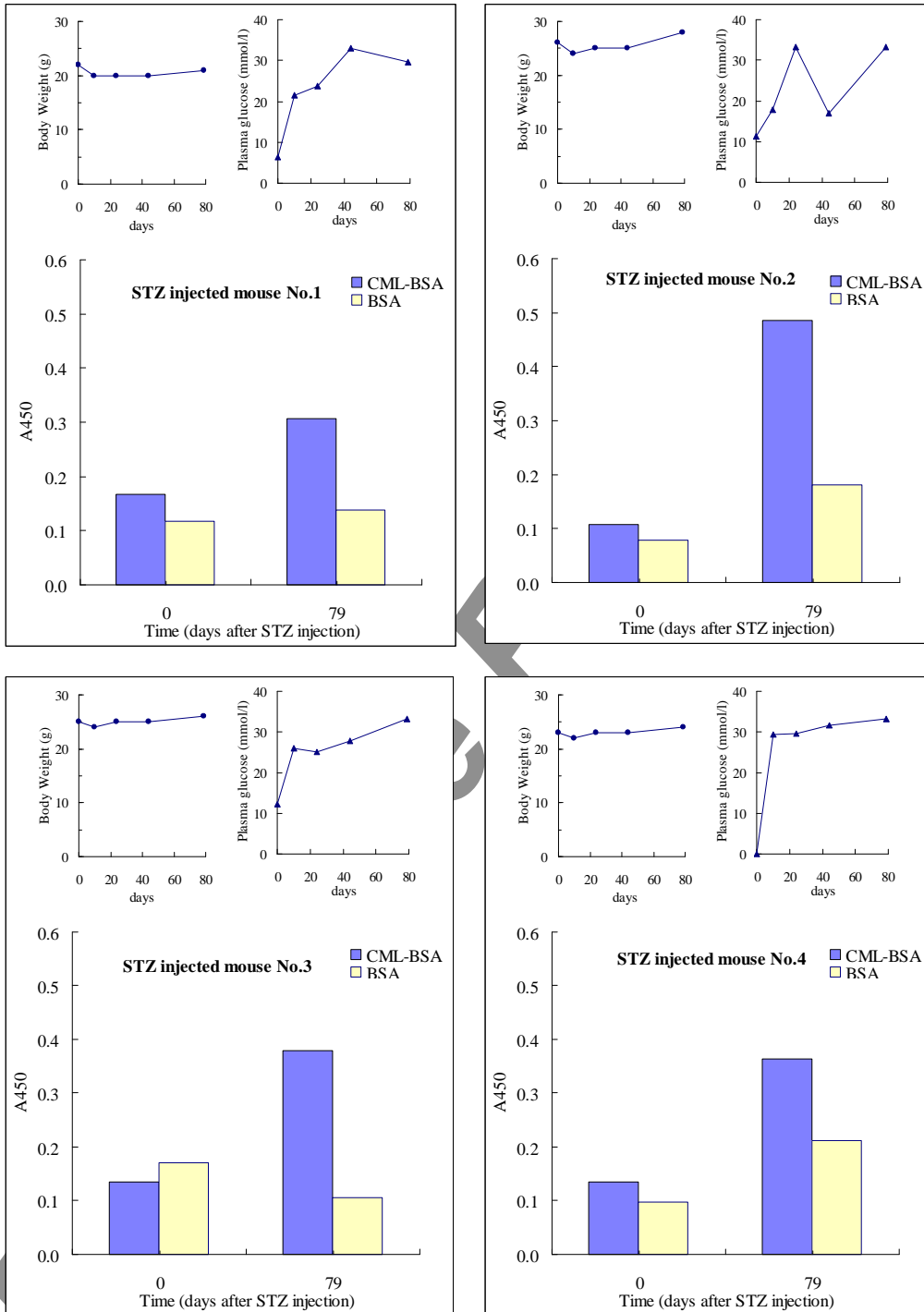Sample Average % Recovery Range
Sera (n=4) 90, 88, 93, 121

5. Linearity

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of anti-CML antibody were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.

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Example of Test Results

Fig.1 Immunoreactivity of plasma of diabetic mice to CML-BSA and BSA.

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Related Products

* **CircuLex CML/N$^\varepsilon$-(Carboxymethyl)lysine ELISA Kit: Cat# CY-8066**
* **CircuLex Anti-CML mouse autoantibody ELISA Kit: Cat# CY-8067**
* **CircuLex Anti-CML human autoantibody ELISA Kit: Cat# CY-8068**
* **CircuLex Anti-CML rat autoantibody ELISA Kit: Cat# CY-8069**

* **CML-BSA/N$^\varepsilon$-(Carboxymethyl)lysine-BSA: Cat# CY-R2052**
* **CML-OVA/N$^\varepsilon$-(Carboxymethyl)lysine -OVA: Cat# CY-R2053**
* **CEL-BSA/N$^\varepsilon$-(Carboxyethyl)lysine-BSA: Cat# CY-R2054**
* **CEL-OVA/N$^\varepsilon$-(Carboxyethyl)lysine-OVA: Cat# CY-R2055**
* **Glucose-AGE-BSA: Cat# CY-R2056**
* **Glucose-AGE-OVA: Cat# CY-R2057**
* **Glyceraldehyde-AGE-BSA: Cat# CY-R2058**
* **Glyceraldehyde-AGE-OVA: Cat# CY-R2059**
* **Glycolaldehyde-AGE-BSA: Cat# CY-R2060**
* **Glycolaldehyde-AGE-OVA: Cat# CY-R2061**
* **Methylglyoxal-AGE-BSA: Cat# CY-R2062**
* **Methylglyoxal-AGE-OVA: Cat# CY-R2063**
* **Glyoxal-AGE-BSA: Cat# CY-R2064**
* **Glyoxal-AGE-OVA: Cat# CY-R2065**
* **CML-HSA/N$^\varepsilon$-(Carboxymethyl)lysine-HSA: Cat# CY-R2066**
* **CEL-HSA/N$^\varepsilon$-(Carboxyethyl)lysine-HSA : Cat# CY-R2067**

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