ELISA Kit for Measuring CML/Nε-(Carboxymethyl)lysine

CircuLex CML/Nε-(Carboxymethyl)lysine ELISA Kit

Cat# CY-8066

Intended Use

The MBL Research Product **CircuLex CML/Nε-(Carboxymethyl)lysine ELISA Kit** is used for the quantitative measurement of CML-adducts in mammalian serum.

Individual users should determine appropriate conditions when using other types of samples.

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.
- Do not expose reagents to excessive light.

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 CML (N$^{\varepsilon}$-(Carboxymethyl)lysine) 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).

Principle of the Assay

The CircuLex CML/N$^{\varepsilon}$-(Carboxymethyl)lysine ELISA Kit employs the quantitative competitive enzyme immunoassay technique. CML-BSA has been pre-coated onto a microplate. Standards or samples and anti-CML-adduct monoclonal antibody MK-5A10 are pipetted into the wells. Any free anti-CML-adduct monoclonal antibody present is bound by the immobilized CML-BSA. After washing away any unbound substances, an HRP conjugated polyclonal 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 inversely proportional to the concentration of CML-adduct. A standard curve is constructed by plotting absorbance values versus CML-adduct concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Mix 50 µL of diluted samples and 50 µL of anti-CML-adduct monoclonal antibody
↓
Add the mixture to each well
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-mouse IgG antibody
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓ Incubate for 10-20 minutes at room temp.
Add 100 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.

Antigen 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.

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

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

Standard Dilution Buffer: One bottle containing 10 mL of 1X buffer; use for standard dilution. Ready to use.

CML-HSA Standard: One vial containing X* µg of lyophilized CML-HSA.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Primary Antibody: One vial containing lyophilized anti-CML-adduct monoclonal antibody.

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

Substrate Reagent: One bottle containing 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

- **Sample preparation microplate:** 96 wells microplate for mixing Primary Antibody and diluted samples.
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Precision repeating pipettor**
- **Orbital microplate shaker**
- **Microcentrifuge and tubes** for sample preparation.
- **Vortex mixer**
- **(Optional) Microplate washer:** 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.
- **(Optional) Software package facilitating data generation and analysis**
- **500 or 1,000 mL graduated cylinder**
- **Reagent reservoirs**
- **Deionized water of the highest quality**
- **Disposable paper towels**

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- 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 in this kit may contain preservatives or other chemicals. 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 human 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.**

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.

Other biological samples: MBL has not tested.
(*e.g.* Remove any particulates by centrifugation and assay immediately or aliquot and store samples at below -70°C. Avoid repeated freeze-thaw cycles. Individual users should determine appropriate conditions when using other types of samples.)

Detailed Protocol

The MBL Research Product **CircuLex CML/Nε-(Carboxymethyl)lysine 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 CML-HSA 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**, **Primary Antibody** and **CML-HSA 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_2O**). Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Reconstitute **Primary Antibody** with X* **mL** of **ddH_2O** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

3. Prepare an appropriate aliquot of Primary Antibody Working Solution by **36-fold** diluting **Primary Antibody** with **Sample Dilution Buffer**. e.g. 3 µL Primary Antibody + 105 µL Sample Dilution Buffer. (60 µL per one well is required for assay.)

4. Reconstitute **CML-HSA Standard** with X* **mL** of **ddH_2O** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 100 µL) to plastic micro-centrifuge tubes and store below -70°C to avoid non-specific adsorption to glass surface and multiple freeze-thaw cycles. The concentration of the reconstituted CML-HSA Standard should be **21 µg/mL**, which is referred to as the **Master Standard** of CML-HSA.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

Prepare Standard Solutions as follows:
Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 7.0 µg/ml standard (Std.1) serves as the highest standard. The **Standard Dilution Buffer** serves as the zero standard (Blank).

	Volume of Standard	Standard Dilution Buffer	Concentration
Std.1	200 µL of Master Standard	400 µL	7.0 µg/mL
Std.2	300 µL of Std. 1 (7.0 µg/mL)	300 µL	3.5 µg/mL
Std.3	300 µL of Std. 2 (3.5 µg/mL)	300 µL	1.75 µg/mL
Std.4	300 µL of Std. 3 (1.75 µg/mL)	300 µL	0.875 µg/mL
Std.5	300 µL of Std. 4 (0.875 µg/mL)	300 µL	0.438 µg/mL
Std.6	300 µL of Std. 5 (0.438 µg/mL)	300 µL	0.219 µg/mL
Std.7	300 µL of Std. 6 (0.219 µg/mL)	300 µL	0.109 µg/mL
Blank	-	300 µL	0 µg/mL

Note: Do not use a Repeating pipette. Change tips for every dilution. Wet tip with Standard Dilution Buffer before dispensing.

Sample Preparation

Dilute samples with **Sample Dilution Buffer**.
• Serum samples may require 4-fold dilution.

Assay Procedure

1. Remove the appropriate number of microtiter wells of **Antigen coated Microplate** 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 **Sample Dilution Buffer** in appropriate wells of a sample preparation microplate.

3. Pipette **60 µL** of **Standard Solutions (Std1-Std7, Blank)** and the **diluted samples** in duplicates, into the appropriate wells of a sample preparation microplate. Pipette **60 µL** of **Primary Antibody Working Solution** into each well. Mix well.

4. Transfer **100 µL** of the mixtures prepared above to each well of **Antigen coated Microplate**.

5. Incubate the wells **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

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

7. Add **100 µL** of **HRP conjugated Detection Antibody** into each well.

8. Incubate the wells **at room temperature (ca.25°C) for 60 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.

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

10. Add **100 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

11. Incubate the wells **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.

12. Add **100 µL** of **Stop Solution** to each well in the same order as the previously added Substrate Reagent.

13. 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 2.0 units for the blank (zero concentration).

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the blank (zero concentrations), perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine the concentration of off-scale samples. The readings at 405 nm should not replace the on-scale readings at 450 nm.

Calculations

Average the duplicate readings for each standard, control and sample. Plot the optical density versus the concentration of standards and draw the best curve. Most microtiter plate readers perform automatic calculations of analyte concentration. The standard curve fits best to a sigmoidal four-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a four-parameter logistic function.

A standard curve is also to be constructed by plotting the absorbance (Y) versus log of the known concentration (X) of standards, using a cubic function. Alternatively, the logit log function can be used to linearize the standard curve (i.e. logit of optical density (Y) is plotted versus log of the known concentration (X) of standards). To determine the concentration of each sample, first find the optical density 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 concentration.

If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Measurement Range

The measurement range is 0.109 µg/mL to 7.0 µg/mL. Any sample reading lower 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 CML-HSA concentration.

Troubleshooting

1. All samples and standards should be assayed 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.

Reagent Stability

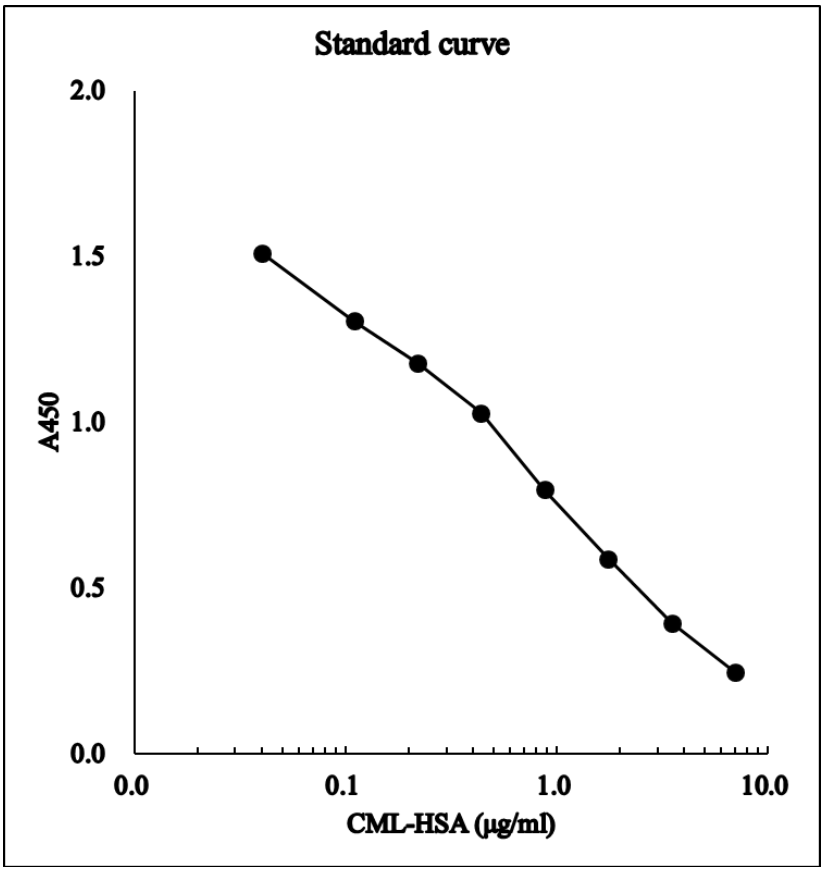
All of the reagents included in the **CircuLex CML/Nε-(Carboxymethyl)lysine ELISA Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date.

Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of CML-HSA giving absorbance lower than mean absorbance plus three standard deviations of the absorbance of Blank: Blank + 3SD Blank) is better than 0.063 µg/mL of sample.

Typical Standard Curve



2. Specificity

The antibodies in the CircuLex CML/N$^{\varepsilon}$-(Carboxymethyl)lysine ELISA Kit are highly specific of CML-adduct, with no detectable cross-reactivity to non-CML-protein that may be present in human serum.

3. Precision

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested seven times on one plate to assess intra-assay precision.

• Intra-assay (Within-Run, n=7) CV=5.2-7.4 %

Corresponding concentration of CML-HSA (µg/mL)

	Sapmle 1	Sapmle 2	Sapmle 3
1	79.61	123.28	259.82
2	70.61	133.71	237.87
3	76.10	123.78	244.76
4	68.68	121.10	260.97
5	68.35	133.72	227.70
6	79.97	133.03	231.16
7	80.83	138.55	269.80
max.	**80.8**	**138.5**	**269.8**
min.	**68.4**	**121.1**	**227.7**
mean	**74.9**	**129.6**	**247.4**
SD	**5.5**	**6.7**	**16.3**
CV(%)	**7.4%**	**5.2%**	**6.6%**

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in three separate assays to assess inter-assay precision.

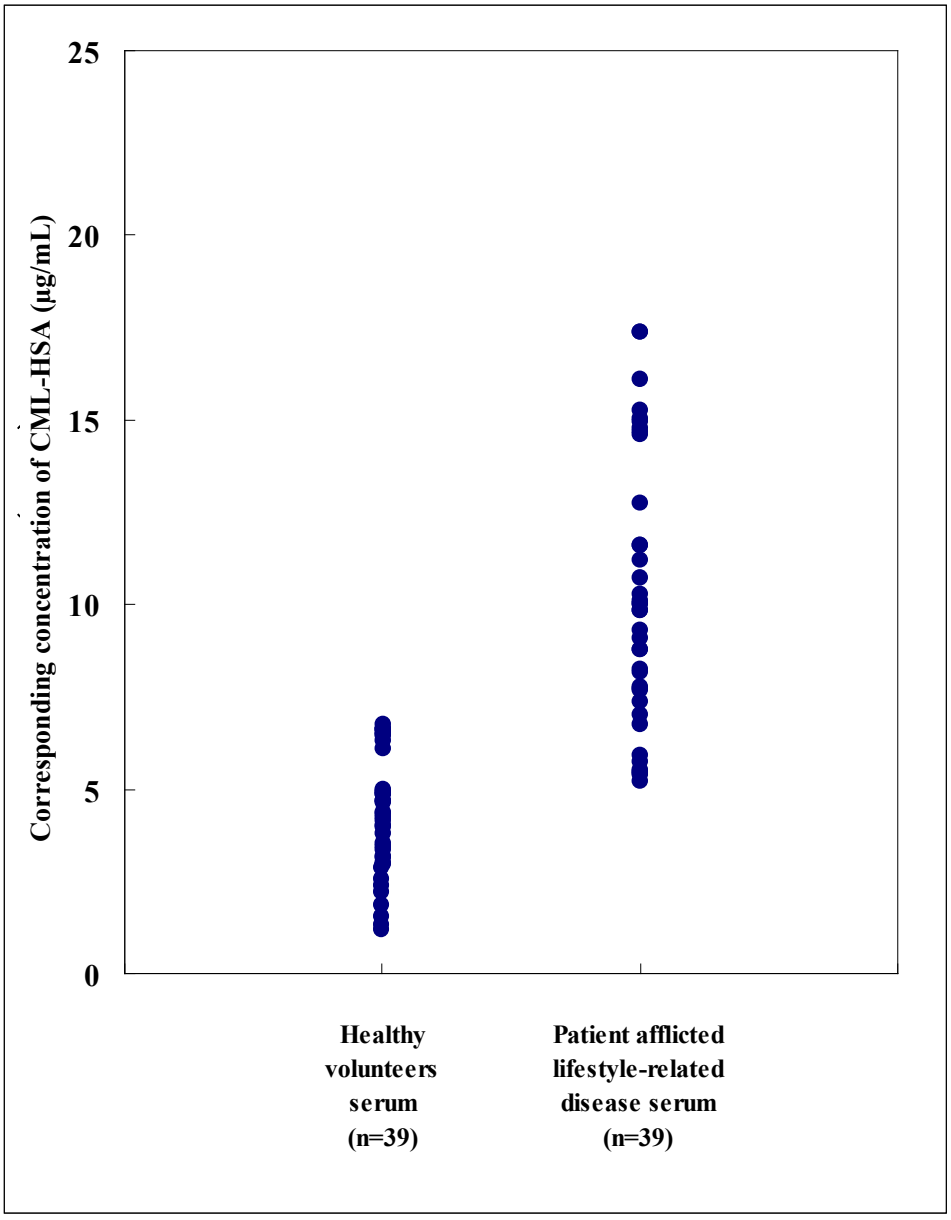
• Inter-assay (Run-to-Run, n=3) CV=4.7-15.2 %

Corresponding concentration of CML-HSA (µg/mL)

	Sapmle 1	Sapmle 2	Sapmle 3
1	60.74	136.80	190.90
2	72.44	131.41	247.41
3	58.04	124.47	255.43
max.	**72.4**	**136.8**	**255.4**
min.	**58.0**	**124.5**	**190.9**
mean	**63.7**	**130.9**	**231.2**
SD	**7.7**	**6.2**	**35.2**
CV(%)	**12.0%**	**4.7%**	**15.2%**

Example of Test Results

Fig.1 Concentrations of CML/N$^{\varepsilon}$-(Carboxymethyl)lysine in patient afflicted lifestyle-related disease and healthy volunteer's serum



References

1. Ikeda K *et al*. N (epsilon)-(carboxymethyl)lysine protein adduct is a major immunological epitope in proteins modified with advanced glycation end products of the Maillard reaction. Biochemistry. 1996 Jun 18;35(24):8075-83.

2. Reddy S *et al*. N epsilon-(carboxymethyl)lysine is a dominant advanced glycation end product (AGE) antigen in tissue proteins. Biochemistry. 1995 Aug 29;34(34):10872-8.

3. Makino H *et al*. Ultrastructure of nonenzymatically glycated mesangial matrix in diabetic nephropathy. Kidney Int. 1995 Aug;48(2):517-26.

4. Suzuki D *et al*. Immunofluorescence staining of renal biopsy samples in patients with diabetic nephropathy in non-insulin-dependent diabetes mellitus using monoclonal antibody to reduced glycated lysine. J Diabetes Complications. 1996 Nov-Dec;10(6):314-9.

5. Imai N *et al*. Histological localization of advanced glycosylation end products in the progression of diabetic nephropathy. Nephron. 1997;76(2):153-60.

6. Murata T *et al*. The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. Diabetologia. 1997 Jul;40(7):764-9.

7. Kume S *et al*. Immunohistochemical and ultrastructural detection of advanced glycation end products in atherosclerotic lesions of human aorta with a novel specific monoclonal antibody. Am J Pathol. 1995 Sep;147(3):654-67.

8. Sakata N *et al*. Immunohistochemical localization of different epitopes of advanced glycation end products in human atherosclerotic lesions. Atherosclerosis. 1998 Nov;141(1):61-75.

9. Sakata N *et al*. Increased advanced glycation end products in atherosclerotic lesions of patients with end-stage renal disease. Atherosclerosis. 1999 Jan;142(1):67-77.

10. Kislinger T *et al*. N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. J Biol Chem. 1999 Oct 29;274(44):31740-9.

For more information, please visit our website at
https://ruo.mbl.co.jp/.

MANUFACTURED BY

MBL MEDICAL & BIOLOGICAL LABORATORIES CO., LTD.
A JSR Life Sciences Company

URL: https://ruo.mbl.co.jp
E-mail: support@mbl.co.jp