ELISA Kit for Measuring Human sRAGE

CircuLex Human sRAGE ELISA Kit

Cat# CY-8083

Intended Use..... 1
Storage..... 1
Introduction..... 2
Principle of the Assay..... 2-3
Materials Provided..... 3
Materials Required but not Provided..... 4
Precautions and Recommendations..... 5
Sample Collection and Storage..... 6
Detailed Protocol..... 7-8
Calculations..... 9
Measurement Range..... 9
Troubleshooting..... 9
Reagent Stability..... 9
Assay Characteristics..... 10-12
Example of Test Results..... 13-14
References..... 15-16
Related Products..... 16

Intended Use

The CycLex Research Product **CircuLex Human sRAGE ELISA Kit** is used for the quantitative measurement of human soluble RAGE (sRAGE) in serum, plasma and other biological media.

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.

Introduction

RAGE is a multi-ligand member of the immunoglobulin superfamily of cell surface molecules that is expressed in a variety of cell lines, including endothelial cells, smooth muscle cells, mononuclear phagocytes, pericytes, neurons, cardiac myocytes, mesangial cells and hepatocytes (1, 2). RAGE interacts with different structures to transmit a signal into the cell and recognizes three-dimensional structures rather than specific amino acid sequences. Therefore, RAGE seems to fulfill the requirements of a pattern-recognition receptor. As a member of the immunoglobulin superfamily, it interacts with a diverse class of ligands, including AGEs (3, 4), HMGB1 (also known as Amphoterin) (5), amyloid β-peptide (6), amyloid A (7), leukocyte adhesion receptors (8), prions (9), Escherichia coli curli operons (10), β-sheet fibrils (11) and several members of the S100 protein superfamily including S100/calgranulins (12). Thus RAGE may have potential involvement in several pathological processes including inflammation, diabetes, Alzheimer's disease (AD), systemic amyloidosis, and tumor growth (13).

Soluble version of RAGE, termed soluble RAGE (sRAGE), created by proteolytical cleavage by matrix metalloproteases can be detected in sera. In addition to sRAGE, another soluble RAGE, as endogenous secretory RAGE (esRAGE) derives from alternative splicing of the RAGE mRNA (14–16) was discovered. Because of the possible neutralization effect of sRAGE, studies have examined the significance of sRAGE serum concentration in patients with various pathological conditions. Decreased level of sRAGE is a biomarker for deficient and/or altered inflammatory control in humans. It was shown that reduced level of sRAGE is associated with higher risk of coronary disease. In Alzheimer disease there is a decrease in serum sRAGE in comparison with patients with vascular dementia and controls. On the other hand, an increased level of serum sRAGE was found in patients with end-stage renal disease and acute lung injury.

Principle of the Assay

The CycLex Research Product **CircuLex Human sRAGE ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human sRAGE is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human sRAGE present. After washing away any unbound substances, an HRP conjugated antibody specific for human sRAGE 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 human sRAGE. A standard curve is constructed by plotting absorbance values versus human sRAGE concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

Summary of Procedure

Add 100 µL of diluted samples to the wells
↓ Incubate for 1 hour at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-human sRAGE antibody
↓ Incubate for 1 hour at room temp.
Wash the wells
↓
Add 100 µL of Substrate Reagent
↓
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.

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 anti-human sRAGE monoclonal antibody (YK-2B4) as a capture antibody.

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

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

Human sRAGE Standard: One vial containing 8 ng of lyophilized recombinant human sRAGE

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-human sRAGE 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 containing 20 mL of 1 N H_2SO_4. Ready to use.

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 450/540 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 residues 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 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.

Plasma: Collect plasma using 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.

Detailed Protocol

The CycLex Research Product **CircuLex Human sRAGE 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 human sRAGE 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 **Human sRAGE Standard**.

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

2. Reconstitute **Human sRAGE Standard** with **1.0 mL** of **Dilution Buffer**. The concentration of the human sRAGE in vial should be **8 ng/mL**, which is referred as a **Master Standard** of human sRAGE.

Prepare Standard Solutions as follows:

Use the **Master Standard** to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 800 pg/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	60 µL of Master Standard (8 ng/mL)	540 µL	800 pg/mL
Std.2	300 µL of Std. 1 (800 pg/mL)	300 µL	400 pg/mL
Std.3	300 µL of Std. 2 (400 pg/mL)	300 µL	200 pg/mL
Std.4	300 µL of Std. 3 (200 pg/mL)	300 µL	100 pg/mL
Std.5	300 µL of Std. 4 (100 pg/mL)	300 µL	50 pg/mL
Std.6	300 µL of Std. 5 (50 pg/mL)	300 µL	25 pg/mL
Std.7	300 µL of Std. 6 (25 pg/mL)	300 µL	12.5 pg/mL
Blank	-	300 µL	0 pg/mL

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

Sample Preparation

- Serum and plasma samples require a 8-fold dilution.
- Other biological samples require neat to appropriate dilution.

Standard Assay Procedure for Human sRAGE

1. Remove the appropriate number of microtiter 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 **100 µL** of **Standard Solutions (Std1-Std7, Blank)** and **diluted samples** in duplicates, into the appropriate wells.

4. Incubate the plate **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 **100 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 **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

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 **100 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 0.25 units for the blank (zero concentration), or 3.0 units for the highest standard concentration. The plate should be monitored at 5-minute intervals for approximately 30 minutes.

Note-3: If the microplate reader is not capable of reading absorbance greater than the absorbance of the highest standard, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine sRAGE 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 and subtract the average zero standard optical density. 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 human sRAGE 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 human sRAGE 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 4-parameter logistic equation. The results of unknown samples can be calculated with any computer program having a 4-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 4-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 12.5 pg/mL to 800 pg/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 human sRAGE concentration.

Troubleshooting

1. The Human sRAGE 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.

Reagent Stability

All of the reagents included in the CycLex Research Product **CircuLex Human sRAGE 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, except the reconstituted sRAGE Standard must be stored at below -70°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack.

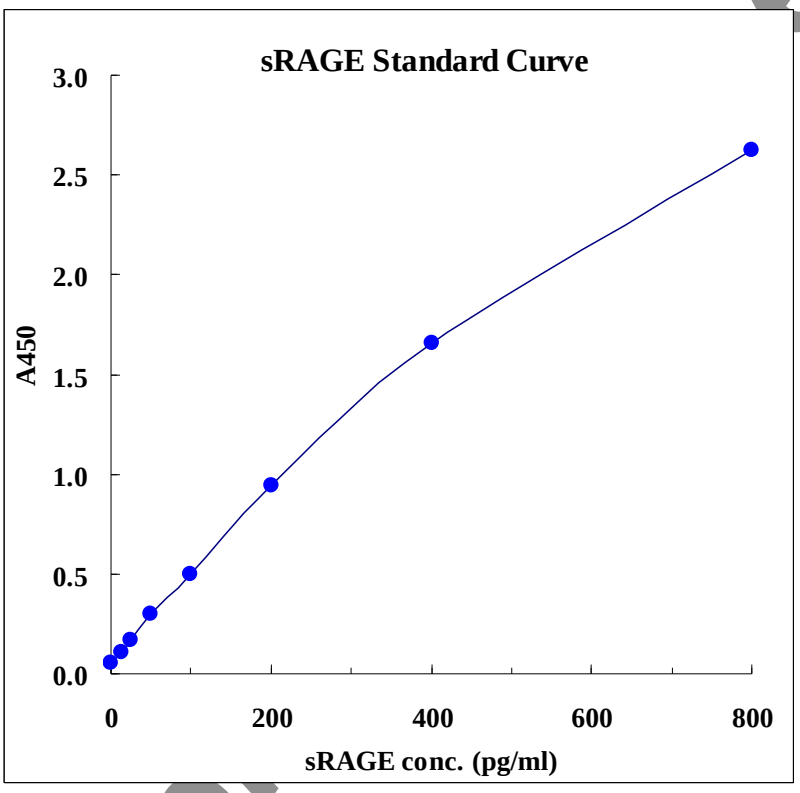
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human sRAGE giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 1.6 pg/ml of sample.

* Standard/Sample Dilution Buffer is pipetted into blank wells.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

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

- Intra-assay (Within-Run, n=8) CV=2.5-5.3 %

Human sRAGE concentration (pg/mL)

	Sample 1	Sample 2	Sample 3	Sample 4
1	222.9	587.0	644.1	923.7
2	218.6	564.0	628.8	819.1
3	196.5	534.7	614.8	898.6
4	224.0	555.6	624.4	807.1
5	213.7	551.0	610.5	855.5
6	197.3	605.6	647.0	909.4
7	215.1	562.2	624.5	842.3
8	214.3	530.3	602.1	818.0
max.	**224.0**	**605.6**	**647.0**	**923.7**
min.	**196.5**	**530.3**	**602.1**	**807.1**
mean	**212.8**	**561.3**	**624.5**	**859.2**
SD	**10.5**	**25.2**	**15.6**	**45.6**
CV (%)	**4.9**	**4.5**	**2.5**	**5.3**

Inter-assay Precision (Precision between assays)

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

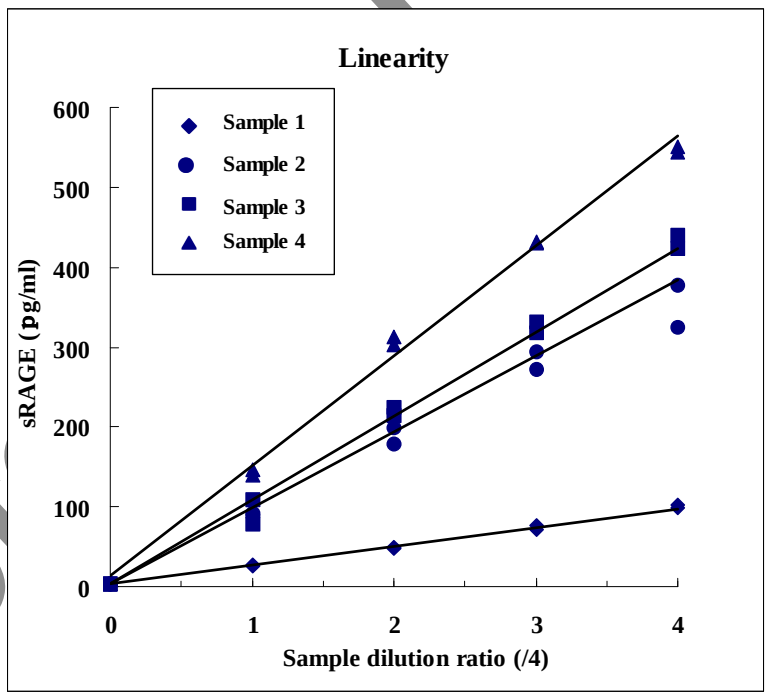
• Inter-assay (Run-to-Run, n=4) CV=4.9-8.0 %

Human sRAGE concentration (ng/mL)

	Sample 1	Sample 2	Sample 3	Sample 4
1	208.3	451.5	669.3	1,022.0
2	192.2	405.5	694.3	868.3
3	196.1	430.5	625.2	910.7
4	179.5	411.0	634.5	865.5
max.	**208.3**	**451.5**	**694.3**	**1,022.0**
min.	**179.5**	**405.5**	**625.2**	**865.5**
mean	**194.0**	**424.6**	**655.8**	**916.6**
SD	**11.9**	**20.9**	**31.9**	**73.2**
CV (%)	**6.1**	**4.9**	**4.9**	**8.0**

3. Linearity

Four serum specimens were diluted with Standard/Sample Dilution Buffer and assayed after dilution. The neat sample is set to 1 (4/4). Please note that all samples including the neat sample are 8-fold diluted as stated in the Assay Procedure. The results are summarized in the figure below.



Example of Test Results

Fig.1 Correlation between the human sRAGE concentrations measured by CircuLex ELISA kit and by competitor ELISA kit

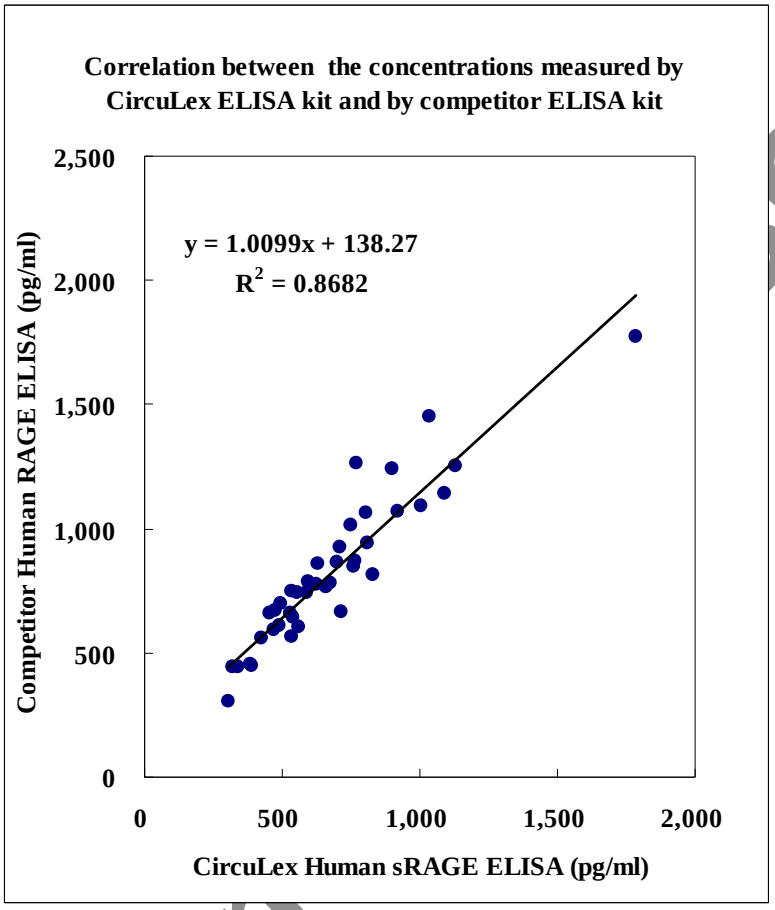
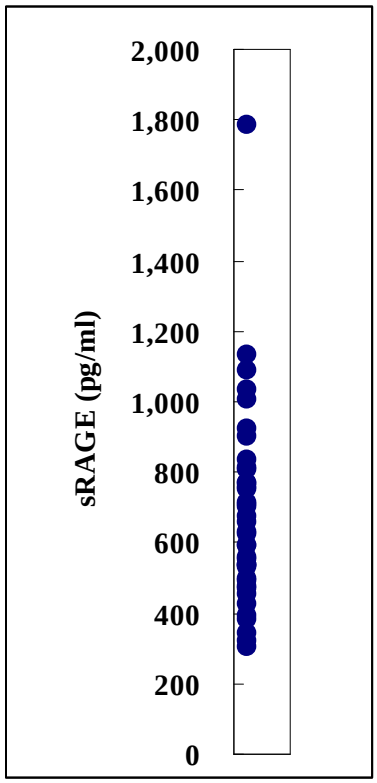


Fig.2 Serum sRAGE level in 40 healthy volunteers



Human sRAGE (n=40)

Max	1783.4
Min	302.9
Mean	676.3

References

1. Neeper M, Schmidt AM, Brett J et al.: Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 1992, **267:** 14998-5004.

2. Brett J, Schmidt AM, Yan SD et al.: Survey of the distribution of a newly characterized receptor for advanced glycation end products in tissues. *Am J Pathol* 1993, **143:** 1699-712.

3. Neeper M, Schmidt AM, Brett J, et al.: Cloning and expression of a cell surface receptor for advanced glycosylation end products of proteins. *J Biol Chem* 1992, **267:** 14998-15004.

4. Schmidt AM, Vianna M, Gerlach M, et al.: Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. *J Biol Chem* 1992, **267:** 14987-14997

5. Hori O, Brett J, Slattery T, Cao R et al.: The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and coexpression of rage and amphoterin in the developing nervous system. *J Biol Chem* 1995, **270:** 25752-25761.

6. Yan SD, Zhu H, Fu J, Yan SF, Roher A et al.: Amyloidbeta peptide-receptor for advanced glycation endproduct interaction elicits neuronal expression of macrophage-colony stimulating factor: a proinflammatory pathway in Alzheimer disease. *Proc Natl Acad Sci U S A* 1997, **94:** 5296-5301.

7. Yan SD, Zhu H, Zhu A, Golabek A, Du H, Roher A, Yu J, Soto C, Schmidt AM, Stern D, et al.: Receptor-dependent cell stress and amyloid accumulation in systemic amyloidosis. *Nat Med* 2000, **6:** 643-651.

8. Chavakis T, Bierhaus A, Al-Fakhri N et al.: The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. *J Exp Med* 2003, **198:** 1507-1515.

9. Sasaki N, Takeuchi M, Chowei H, Kikuchi S et al.: Advanced glycation end products (AGE) and their receptor (RAGE) in the brain of patients with Creutzfeldt-Jakob disease with prion plaques. *Neurosci Lett* 2002, **326:** 117-120.

10. Chapman MR, Robinson LS, Pinkner JS et al.: Role of Escherichia coli curli operons in directing amyloid fiber formation. *Science* 2002, **295:** 851-855

11. Bierhaus A, Humpert PM, Morcos M, et al.: Understanding RAGE, the receptor for advanced glycation end products. *J Mol Med* 2005, **83:** 876- 886.

12. Hofmann MA, Drury S, Fu C, Qu W et al.: RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell* 1999, **97:** 889-901.

13. Stern DM, Yan SD, Yan SF, and Schmidt AM: Receptor for advanced glycation endproducts (RAGE) and the complications of diabetes. *Ageing Res Rev.* 2002, **1:** 1-15.

14. Schlueter, C., Hauke, S., Flohr, A. M., Rogalla, P., and Bullerdiek, J.: Biochim. Biophys. Acta 2003, **1630:** 1–6

15. Yonekura, H., Yamamoto, Y., Sakurai, S., Petrova, R. G., Abedin, M. J., Li, H., Yasui, K., Takeuchi, M., Makita, Z., Takasawa, S., Okamoto, H., Watanabe, T., and Yamamoto, H.: Biochem. J. 2003, **370:** 1097–1109

16. Malherbe, P., Richards, J. G., Gaillard, H., Thompson, A., Diener, C., Schuler, A., and Huber, G. Brain Res.: Mol. Brain Res. 1999, **71:** 159–170

Related Products

*** CircuLex S100A12/EN-RAGE ELISA Kit: Cat# CY-8058**
*** Human S100A12: Cat# CY-R2262-G**
*** Human S100A12: Cat# CY-R2262-H**
*** Human S100A12 Low Endotoxin: Cat# CY-R2462-G**
*** CML-BSA/Nε-(carboxymethyl) Lysine-BSA: Cat# CY-R2052**
*** CML-OVA/Nε-(carboxymethyl) Lysine-OVA: Cat# CY-R2053**
*** CEL-BSA/Nε-(carboxyethyl) Lysine-BSA: Cat# CY-R2054**
*** CEL-OVA/Nε-(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/Nε-(carboxymethyl) Lysine-HSA: Cat# CY-R2066**
*** CEL-HSA/Nε-(carboxyethyl) Lysine-HSA: Cat# CY-R2067**

PRODUCED BY

CycLex Co., Ltd.
1063-103 Terasawaoka
Ina, Nagano 396-0002
Japan
Fax: +81-265-76-7618
e-mail: info@cyclex.co.jp
URL: http://www.cyclex.co.jp