ELISA Kit for Measuring Human S100A12/EN-RAGE

Circulex S100A12/EN-RAGE ELISA Kit Ver.2

Cat# CY-8058V2

Intended Use

The MBL Research Product **Circulex S100A12/EN-RAGE ELISA Kit Ver.2** is used for the quantitative measurement of human S100A12/EN-RAGE in serum and plasma.

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.
- Don't expose reagents to excessive light.

Introduction

Members of the S100 protein family are low molecular mass acidic proteins characterized by cell-type-specific expression and the presence of 2 EF-hand calcium-binding domains. The calgranulins are S100 proteins that are expressed in neutrophils, and are abundant in infiltrating monocytes and granulocytes under conditions of chronic inflammation.

Hofmann et al. (1999) reported that RAGE is a central cell surface receptor for S100A12, which they referred to as EN-RAGE (Extracellular Newly identified RAGE-binding protein), and related members of the S100/calgranulin superfamily. Interaction of EN-RAGE (S100A12) with cellular RAGE on endothelium, mononuclear phagocytes, and lymphocytes triggered cellular activation, with generation of key proinflammatory mediators. In murine models, blockade of ENRAGE/RAGE quenched delayed-type hypersensitivity and inflammatory colitis by arresting activation of central signaling pathways and expression of inflammatory gene mediators (1).

S100A12 was also isolated as proteins binding to three different anti-allergic drugs, amlexanox, cromolyn and tranilast, by drug-affinity chromatography (2). This finding implies that these three compounds might interact with these proteins when working as anti-allergic drugs.

S100A12 serum concentrations indicate neutrophil activation in JRA (3), cystic fibrosis (4), Kawasaki disease (5) and Crohn's disease (6). Its function as a proinflammatory factor secreted by activated neutrophils makes this protein a potential target for future therapies.

Principle of the Assay

The Circulex S100A12/EN-RAGE ELISA Kit Ver.2 employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for S100A12/EN-RAGE has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any S100A12/EN-RAGE present. After washing away any unbound substances, an HRP conjugated monoclonal antibody specific for S100A12/EN-RAGE 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 S100A12/EN-RAGE. A standard curve is constructed by plotting absorbance values versus S100A12/EN-RAGE concentrations of calibrators, and concentrations of unknown samples are determined using this standard curve.

The Circulex S100A12/EN-RAGE ELISA Kit Ver.2 is designed to measure the concentration of human S100A12/EN-RAGE from serum, plasma, and other biological media.

Summary of Procedure

Add 100 µL of diluted sample to the wells
↓ Incubate for 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-S100A12 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.

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-S100A12/EN-RAGE monoclonal antibody as a capture antibody.

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

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

Human S100A12 Standard: One vial containing X* ng of lyophilized recombinant human S100A12/EN-RAGE.
***The amount is changed depending on lot. See the real "User's Manual" included in the kit box.**

HRP conjugated Detection Antibody: One bottle containing 12 mL of HRP (horseradish peroxidase) conjugated anti-S100A12/EN-RAGE monoclonal antibody.

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 200-1,000 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.

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

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 S100A12/EN-RAGE ELISA Kit Ver.2** 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 S100A12 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 S100A12 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 **Human S100A12 Standard** with X* **mL** of **ddH_2O**. The concentration of the human S100A12 in vial should be **25.6 ng/mL**, which is referred as a **Master Standard** of human S100A12.
***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 1,280 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	50 µL of Master Standard (25.6 ng/mL)	950 µL	1,280 pg/mL
Std.2	300 µL of Std. 1 (1,280 pg/mL)	300 µL	640 pg/mL
Std.3	300 µL of Std. 2 (640 pg/mL)	300 µL	320 pg/mL
Std.4	300 µL of Std. 3 (320 pg/mL)	300 µL	160 pg/mL
Std.5	300 µL of Std. 4 (160 pg/mL)	300 µL	80 pg/mL
Std.6	300 µL of Std. 5 (80 pg/mL)	300 µL	40 pg/mL
Std.7	300 µL of Std. 6 (40 pg/mL)	300 µL	20 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

Dilute samples with **Dilution Buffer**.
- The recommended dilution for serum and plasma samples is 100- to 1,000-fold.
- Users should determine appropriate dilution ratio of other biological samples.

Assay Procedure

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 sample 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 60 minutes**, 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 60 minutes**, 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 0.2 units for the blank (zero concentration), or 2.5 units for the highest standard concentration.

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 S100A12/EN-RAGE 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 S100A12 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 S100A12 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 20 pg/mL to 1,280 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 S100A12 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 MBL Research Product **Circulex S100A12/EN-RAGE ELISA Kit Ver.2** 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 Human S100A12 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.

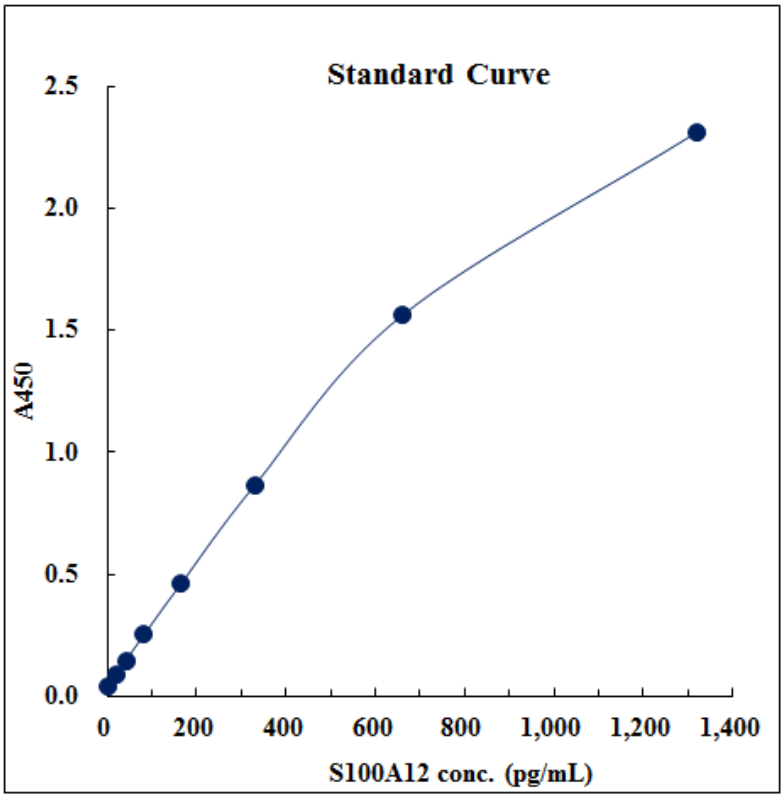
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of S100A12/EN-RAGE giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 8.2 pg/mL of sample.

* Dilution Buffer was pipetted into blank wells.

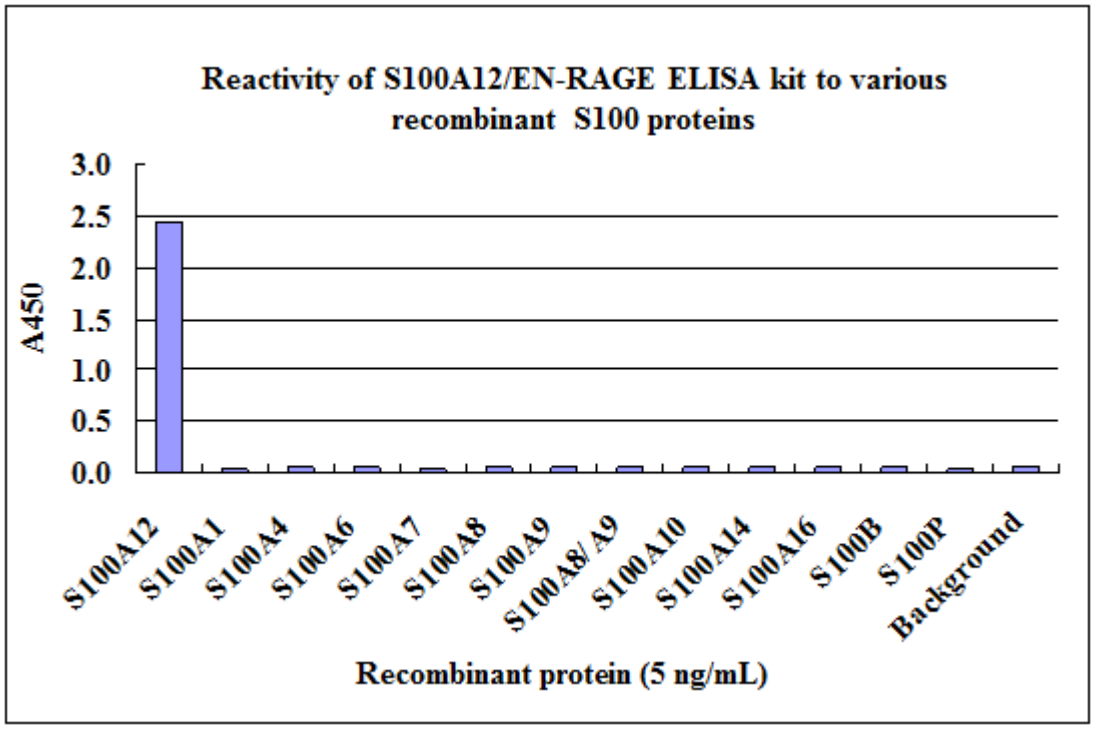
Typical standard curve



2. Specificity

The antibodies in the Circulex S100A12/EN-RAGE ELISA Kit Ver.2 only react with human S100A12 and without detectable cross-reactivities to other human S100 proteins as indicated below.

Reactivity to various recombinant S100 proteins



3. Precision

Intra-assay Precision (Precision within an assay)

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

• Intra-assay (Within-Run, n=8) CV=3.4 - 5.3 %

Sample No.	Sample 1	Sample 2	Sample 3
1	65.348	184.713	211.894
2	67.521	180.957	223.358
3	68.874	192.349	209.243
4	68.245	193.350	216.801
5	70.797	201.191	241.216
6	68.451	192.315	211.727
7	70.764	184.471	219.324
8	73.108	210.559	237.067
max.	73.108	210.559	241.216
min.	65.348	180.957	209.243
mean	69.139	192.488	221.329
SD	2.221	9.088	11.175
CV(%)	3.4	5.0	5.3

Inter-assay Precision (Precision between assays)

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

• Inter-assay (Run-to-Run, n=4) CV=5.3 - 6.2 %

Sample No.	Sample 1	Sample 2	Sample 3
1	67.498	187.848	215.323
2	66.213	164.344	212.425
3	74.901	179.144	226.606
4	73.915	179.800	238.224
max.	74.901	187.848	238.224
min.	66.213	164.344	212.425
mean	70.632	177.784	223.145
SD	4.410	9.795	11.768
CV(%)	6.2	5.5	5.3

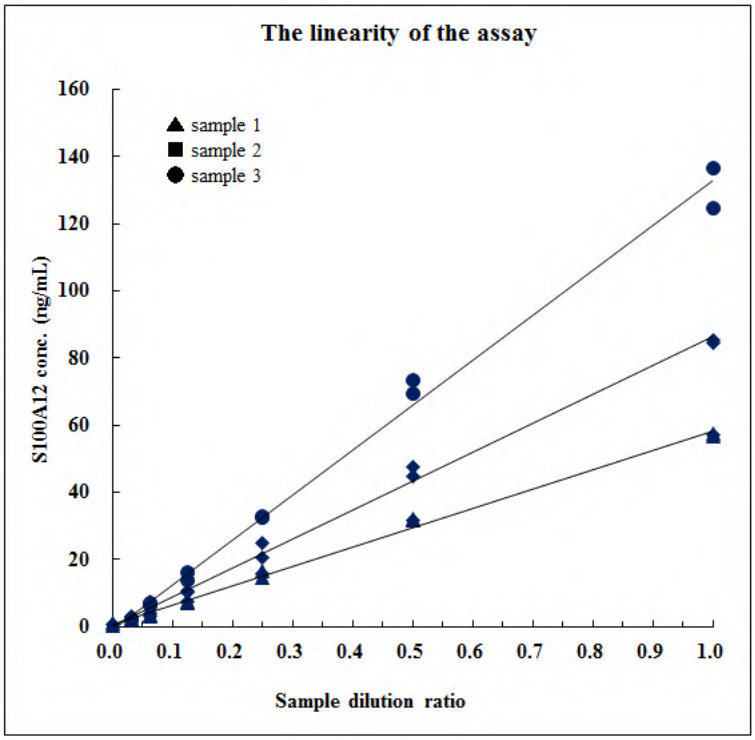
4. Spiking recover

Serum samples were spiked with different amounts of S100A12 and assayed. The recovery of S100A12 spiked to levels throughout the range of the assay was evaluated.

Sample	Spiked Concentration (pg/mL)	Observed Concentration (pg/mL)	Expected Concentration (pg/mL)	Recovery (%)
L	0	109.35	-	-
	100	196.64	209.35	87.3
	200	289.29	309.35	90.0
	400	449.80	509.35	85.1
M	0	106.99	-	-
	100	203.29	206.99	96.3
	200	292.55	306.99	92.8
	400	463.05	506.99	89.0
H	0	212.39	-	-
	100	334.24	312.39	121.8
	200	431.77	412.39	109.7
	400	611.92	612.39	99.9

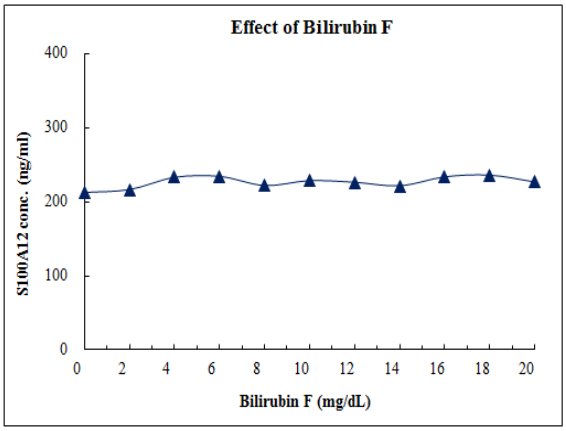
5. Linearity

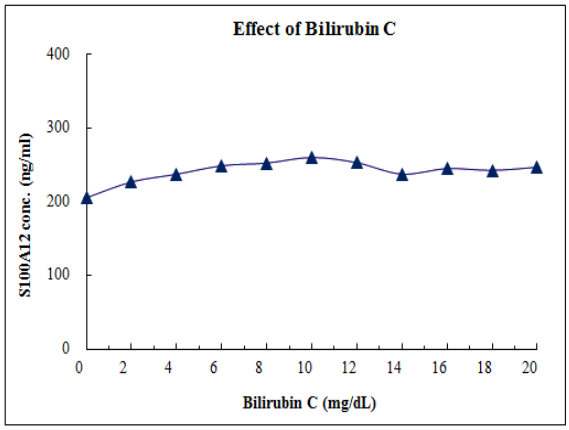
To assess the linearity of the assay, samples containing and/or spiked with high concentrations of S100A12 were serially diluted with the Dilution Buffer to produce samples with values within the dynamic range of the assay.

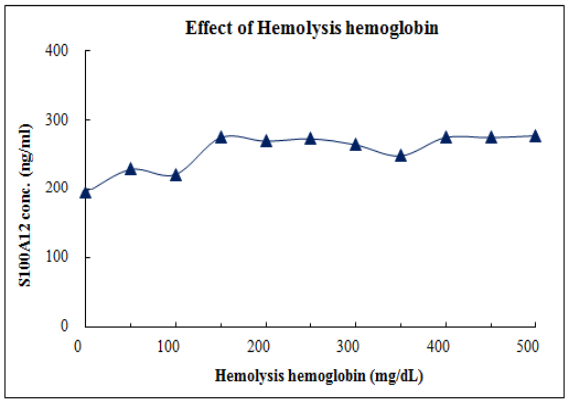


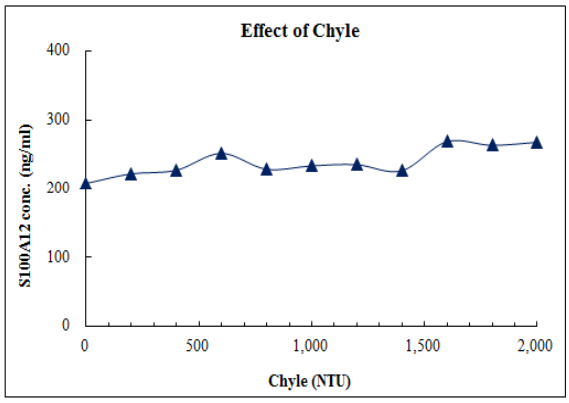
6. Effects of potentially interfering substances in serum on this ELISA Kit

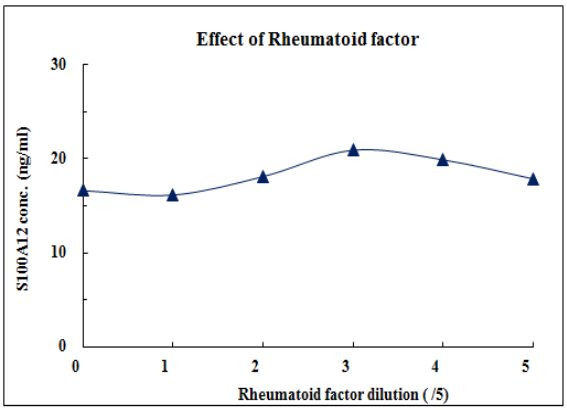
Potentially interfering substances in serum, Bilirubin F, Bilirubin C, Hemolysis hemoglobin, Chyle, and Rheumatoid factor, were added to serum samples at various concentrations. None of the substances at the concentrations tested interfered in the assay.





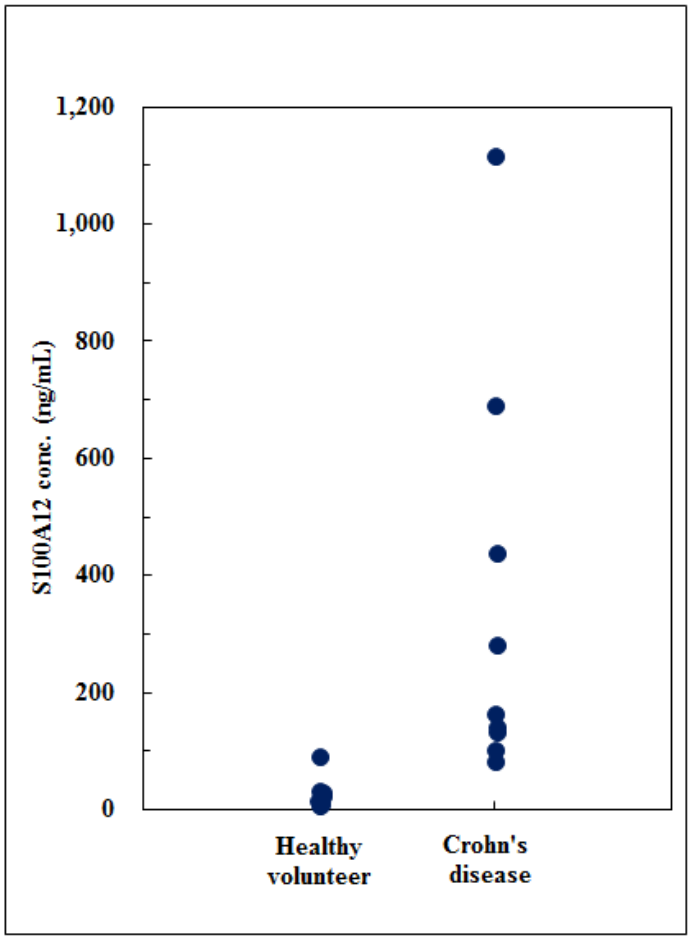






Example of Test Results

Fig.1 Human S100A12 concentrations in sera from Crohn's disease patients (n=10) and healthy volunteers (n=8).



References

1. Hofmann, M. A.; Drury, S.; Fu, C.; Qu, W.; Taguchi, A.; Lu, Y.; Avila, C.; Kambham, N.; Bierhaus, A.; Nawroth, P.; Neurath, M. F.; Slattery, T.; Beach, D.; McClary, J.; Nagashima, M.; Morser, J.; Stern, D.; Schmidt, A. M. : RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell*. **97:** 889-901, 1999

2. Shishibori T, Oyama Y, Matsushita O, Yamashita K, Furuichi H, Okabe A, Maeta H, Hata Y, Kobayashi R.: Three distinct anti-allergic drugs, amlexanox, cromolyn and tranilast, bind to S100A12 and S100A13 of the S100 protein family. *Biochem J.* **338:** 583-9, 1999

3. Foell D, Wittkowski H, Hammerschmidt I, Wulffraat N, Schmeling H, Frosch M, Horneff G, Kuis W, Sorg C, Roth J. Monitoring neutrophil activation in juvenile rheumatoid arthritis by S100A12 serum concentrations. *Arthritis Rheum.* **50:** 1286-95, 2004

4. Foell D, Seeliger S, Vogl T, Koch HG, Maschek H, Harms E, Sorg C, Roth J. : Expression of S100A12 (EN-RAGE) in cystic fibrosis. *Thorax.* **58:** 613-7, 2003

5. Foell D, Ichida F, Vogl T, Yu X, Chen R, Miyawaki T, Sorg C, Roth J.: S100A12 (EN-RAGE) in monitoring Kawasaki disease. *Lancet.* **361:** 1270-2, 2003

6. Neutrophil derived human S100A12 (EN-RAGE) is strongly expressed during chronic active inflammatory bowel disease. *Gut.* **52:** 847-53, 2003

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