ELISA Kit for Measuring Human Calprotectin

CircuLex Human Calprotectin ELISA Kit

Cat# CY-8107

Intended Use

The MBL Research Product **CircuLex Human Calprotectin ELISA Kit** is used for the quantitative measurement of human calprotectin in serum, plasma, milk, tear, saliva, sweat, and urine.

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

S100A9 and S100A8 belong to the low molecular mass calcium-binding S100 protein family (1), they are composed of two distinct helix-loop-helix motifs (EF-hands) flanked by hydrophobic regions at either terminus and separated by a central hinge region. In human, S100A9 is usually co-expressed with S100A8, and they form heterodimeric complexes, which was named as calprotectin (2).

Although a number of possible functions for calprotectin, including antimicrobial activity, have been proposed, the exact role of these proteins in cell metabolism is still unclear. In human, they have been associated with several inflammatory diseases (3): phagocytes expressing S100A9 belong to the early infiltrating cells and dominate acute inflammatory lesions; in addition, elevated serum levels of calprotectin have been found in patients suffering from a number of inflammatory disorders including cell arteritis (4), cystic fibrosis, rheumatoid arthritis, dermatoses, chronic inflammatory bowel disease (IBD), chronic bronchitis (3), some malignancies and autoimmune diseases (5,6). It could be demonstrated with human monocytes that both S100A9 protein and S100A8 proteins are secreted by an energy-consuming pathway, which is dependent on an intact microtubule network and involves protein kinase C (7).

Fecal calprotectin is a marker for inflammatory gastrointestinal as well as neoplastic diseases. It is often difficult to distinguish between IBD and irritable bowel syndrome (IBS). This leads in many cases to extensive and unnecessary colonoscopic examinations. Fecal calprotectin may be a useful marker for discriminating between patients with IBD and IBS (8). In addition, fecal calprotectin levels correlate significantly with histologic and endoscopic assessment of disease activity in Crohn's disease and ulcerative colitis.

Principle of the Assay

The MBL Research Product **CircuLex Human Calprotectin ELISA Kit** employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human calprotectin is pre-coated onto a microplate. Standards and samples are pipetted into the wells and the immobilized antibody binds any human calprotectin present. After washing away any unbound substances, an HRP conjugated antibody specific for human calprotectin 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 calprotectin. A standard curve is constructed by plotting absorbance values versus human calprotectin 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 60 minutes at room temp.
Wash the wells
↓
Add 100 µL of HRP conjugated anti-human calprotectin 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-human calprotectin monoclonal antibody as a capture antibody.

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

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

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

100x HRP conjugated Detection Antibody: One bottle containing 200 uL of HRP (horseradish peroxidase) conjugated anti-human calprotectin monoclonal antibody. Ready to use.

Conjugate Dilution Buffer: One bottle containing 12 mL of Conjugate Dilution Buffer.

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**
- **(Optional) Microplate washer:** Manual washing is possible but not preferable.
- **Plate reader:** capable of measuring absorbance in 96-well plates at dual wavelengths of 450/540 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 1000 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 Substrate Solution which contains hydrogen peroxide.
- Avoid contact with Stop Solution which contains Sulfuric Acid.
- 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 below -70°C for extended periods of time. Avoid repeated freeze-thaw cycles.

Plasma: Collect plasma using EDTA-2Na or heparin as the anticoagulant. If possible, collect the plasma into a mixture of EDTA-2Na 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 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.

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

Detailed Protocol

The MBL Research Product **CircuLex Human Calprotectin 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 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**, **100x HRP conjugated Detection Antibody** and **Human Calprotectin 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. Mix well. Store at 4°C for two weeks or -20°C for long-term storage.

2. Prepare HRP conjugated Detection Antibody by 100-fold diluting **100X HRP-conjugated Detection Antibody** with Conjugate Dilution Buffer at the time of assay. Prepare appropriate volume for your assay. Discard any unused HRP-conjugated Detection Antibody after use.

3. Reconstitute **Human Calprotectin Standard** with X* **mL** of **Dilution Buffer** by gently mixing. After reconstitution, immediately dispense it in small aliquots (e.g. 200 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 Human FABP5 Standard should be **640 ng/mL**, which is referred to as the **Master Standard** of human calprotectin.
***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 32 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	100 µL of Master Standard (640 ng/mL)	900 µL	64 ng/mL
Std.2	300 µL of Std. 1 (64 ng/mL)	300 µL	32 ng/mL
Std.3	300 µL of Std. 2 (32 ng/mL)	300 µL	16 ng/mL
Std.4	300 µL of Std. 3 (16 ng/mL)	300 µL	8 ng/mL
Std.5	300 µL of Std. 4 (8 ng /mL)	300 µL	4 ng/mL
Std.6	300 µL of Std. 5 (4 ng/mL)	300 µL	2 ng/mL
Std.7	300 µL of Std. 6 (2 ng /mL)	300 µL	1 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. Discard any unused Standard Solutions after use.

Sample Preparation

Dilute samples with **Dilution Buffer**.

- Serum and plasma samples may require 500- to 1,000-fold dilution.
- Biological samples require appropriate dilutions.
  Milk: ~50-fold, Tear: ~100-fold, Saliva: ~5,000-fold, Sweat: ~500-fold, Urine: ~100-fold.

Standard Assay Procedure for Human Calprotectin

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 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 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 aspiration or decantation. 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.

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 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, and subtract the optical density of the average zero standard. 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 1 ng/mL to 64 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 concentration of the sample.

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 human Calprotectin 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 Human Calprotectin 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.

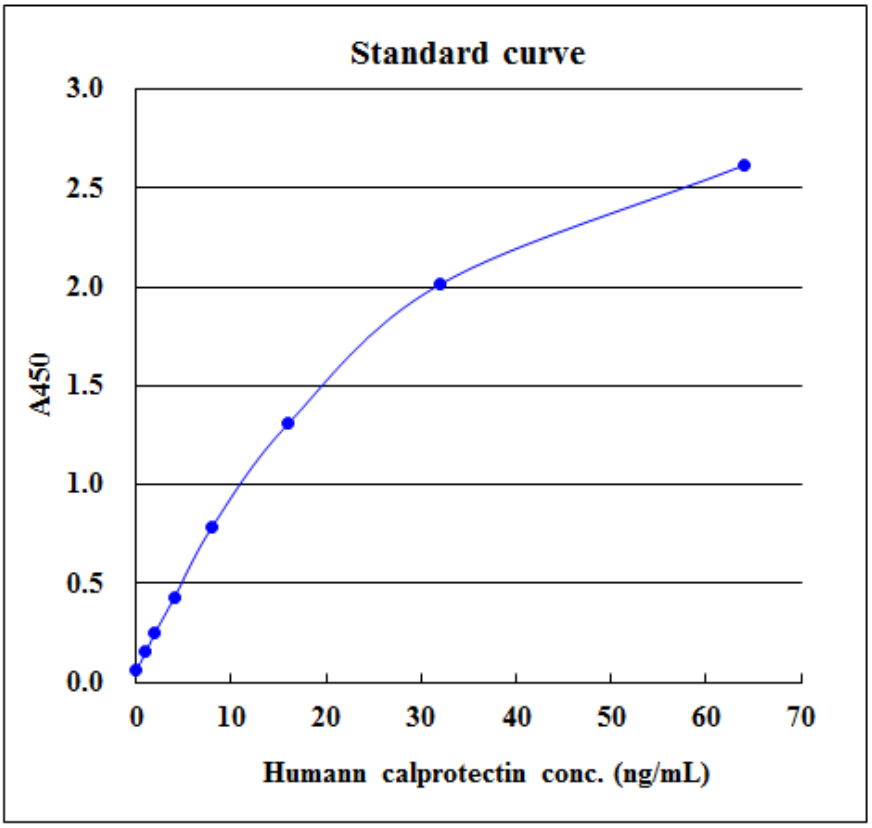
Assay Characteristics

1. Sensitivity

The limit of detection (defined as such a concentration of human calprotectin giving absorbance higher than mean absorbance of blank* plus three standard deviations of the absorbance of blank: A blank + 3SD blank) is better than 0.145 ng/mL of sample.

* Dilution Buffer was pipetted into blank wells.

Typical Standard Curve



2. Precision

Intra-assay Precision (Precision within an assay)

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

• Intra-assay (Within-Run, n=16) CV=3.2-5.7 %

*Sample: Human serum

Human calprotectin conc. (µg/mL)

	Sample 1	Sample 2	Sample 3	Sample 4
1	1.63	8.86	11.79	16.15
2	1.67	8.59	11.67	16.65
3	1.63	8.88	11.92	20.68
4	1.58	9.41	12.20	17.66
5	1.71	9.04	12.06	17.39
6	1.54	8.73	11.81	17.22
7	1.61	8.75	12.23	17.87
8	1.63	9.28	12.33	18.43
9	1.42	8.39	12.43	17.80
10	1.49	8.53	12.78	18.07
11	1.52	9.05	12.06	18.23
12	1.60	9.19	12.98	17.17
13	1.64	8.97	12.48	18.15
14	1.54	8.95	11.79	16.75
15	1.63	8.63	12.45	18.37
16	1.63	9.10	12.92	18.17
MAX.	1.71	9.41	12.98	20.68
MIN.	1.42	8.39	11.67	16.15
MEAN	1.59	8.90	12.24	17.80
S.D.	0.07	0.28	0.41	1.02
C.V.	**4.6%**	**3.2%**	**3.4%**	**5.7%**

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=4.6-8.7 %

*Sample: Human serum

Human calprotectin conc. (µg/mL)

	Sample 1	Sample 2	Sample 3	Sample 4
1	1.45	7.89	10.35	15.09
2	1.71	7.58	9.57	16.04
3	1.75	7.74	10.59	16.32
4	1.63	8.93	11.80	16.85
MAX.	1.75	8.93	11.80	16.85
MIN.	1.45	7.58	9.57	15.09
MEAN	1.63	8.04	10.57	16.08
S.D.	0.14	0.61	0.92	0.74
C.V.	**8.3%**	**7.6%**	**8.7%**	**4.6%**

3. Spiking Recovery

Recombinant human calprotectin was added to three samples* at different concentrations.

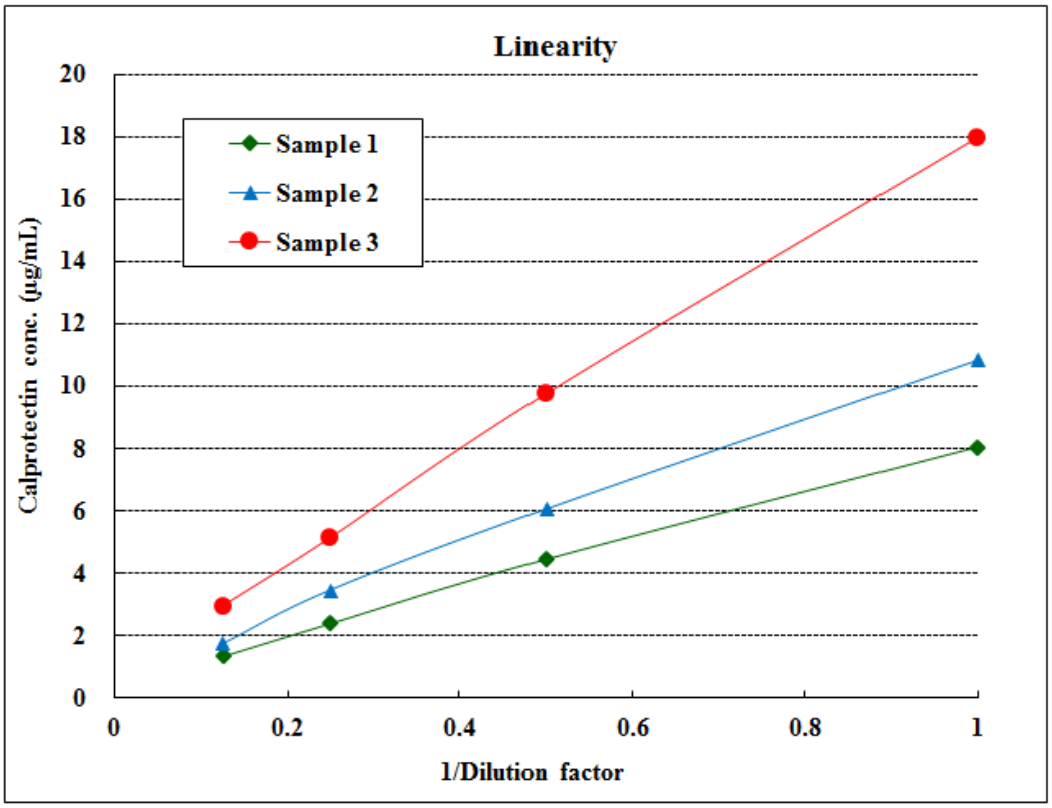
*Sample: Diluted human serum

Sample	Spiked Concentration (ng/ml)	Ovserved Concentration (ng/ml)	Expected Concentration (ng/ml)	Recovery (%)
Low	0	7.1	-	-
	4	9.5	11.1	116.5
	6	11.5	13.1	114.3
	8	14.1	15.1	107.3
Medium-1	0	11.1	-	-
	4	15.5	15.1	96.9
	8	18.6	17.1	91.7
	10	22.9	19.1	83.1
Medium-2	0	17.9	-	-
	4	23.1	21.9	95.0
	6	26.6	23.9	89.8
	8	31.9	25.9	81.3

4. Linearity

Three samples* were diluted with Dilution Buffer and assayed after dilution. The neat sample was set to 1. The results are summarized in the figure below.

*Sample: human serum



Example of Test Results

Fig.1 Human calprotectin concentration in sera from Crohn's disease patients, CRP positive patients, and healthy volunteers

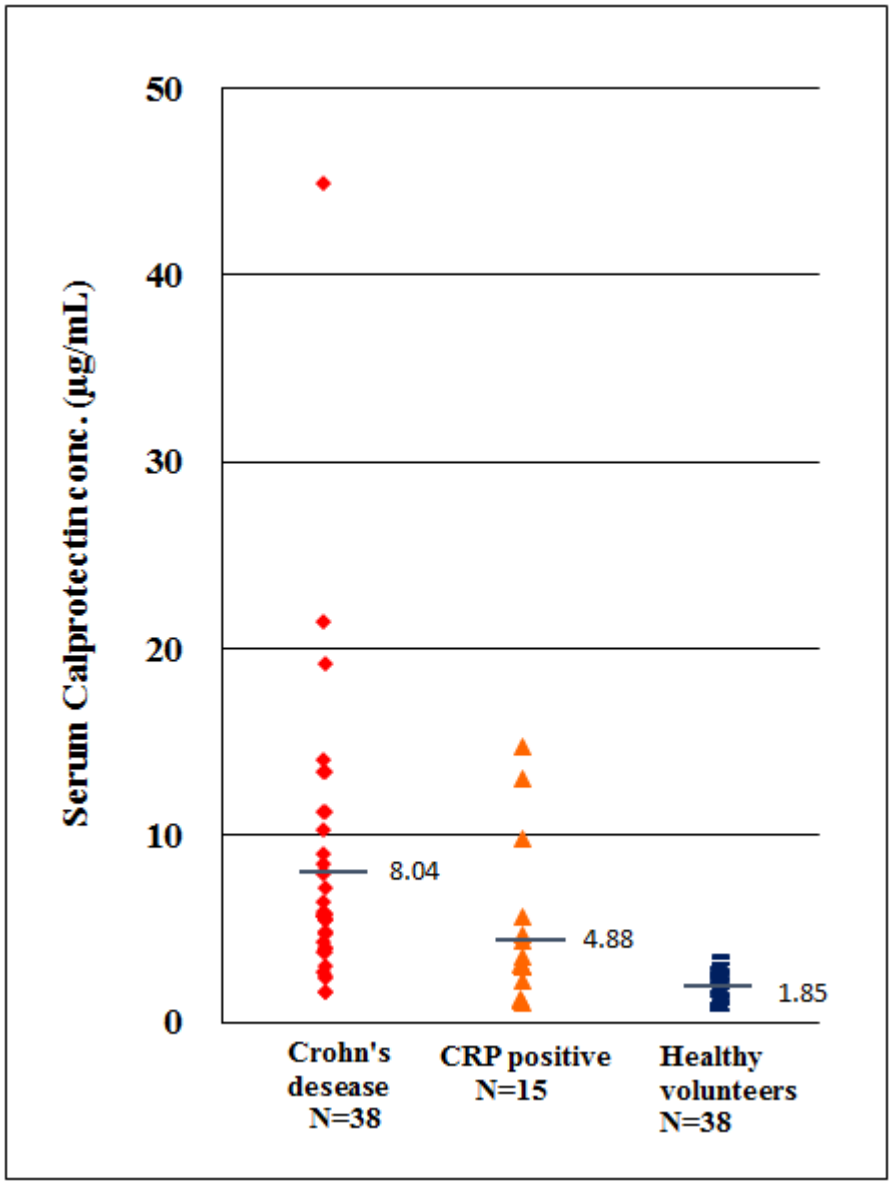


Fig.2 Correlation between MBL's "CircuLex Human Calprotectin ELISA Kit" and company B's counterpart in sera from Crohn's disease and CRP positive patients.

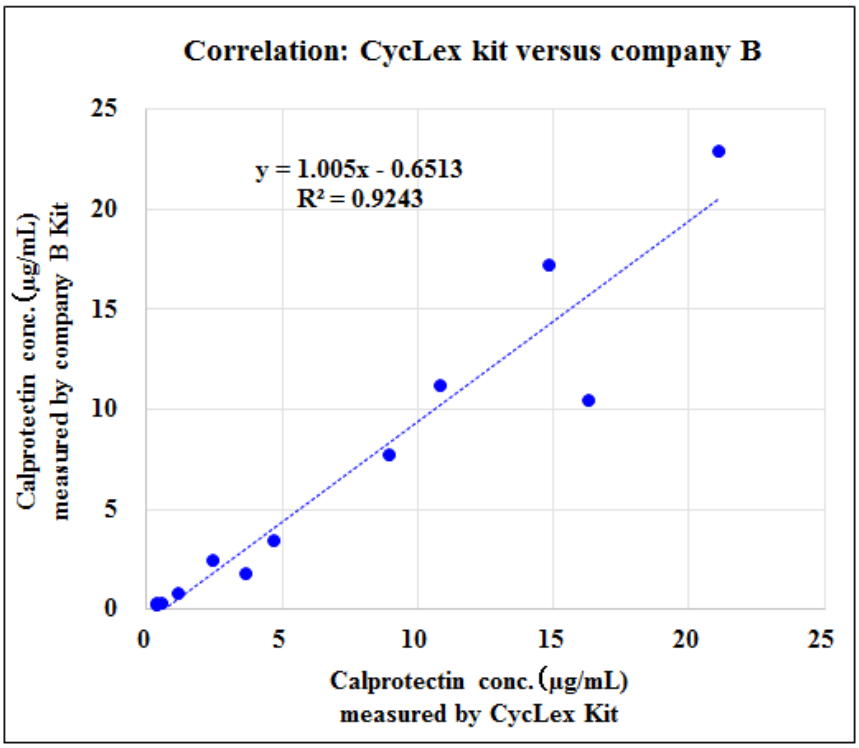


Fig.3 Human calprotectin concentration in tear samples from healthy volunteers

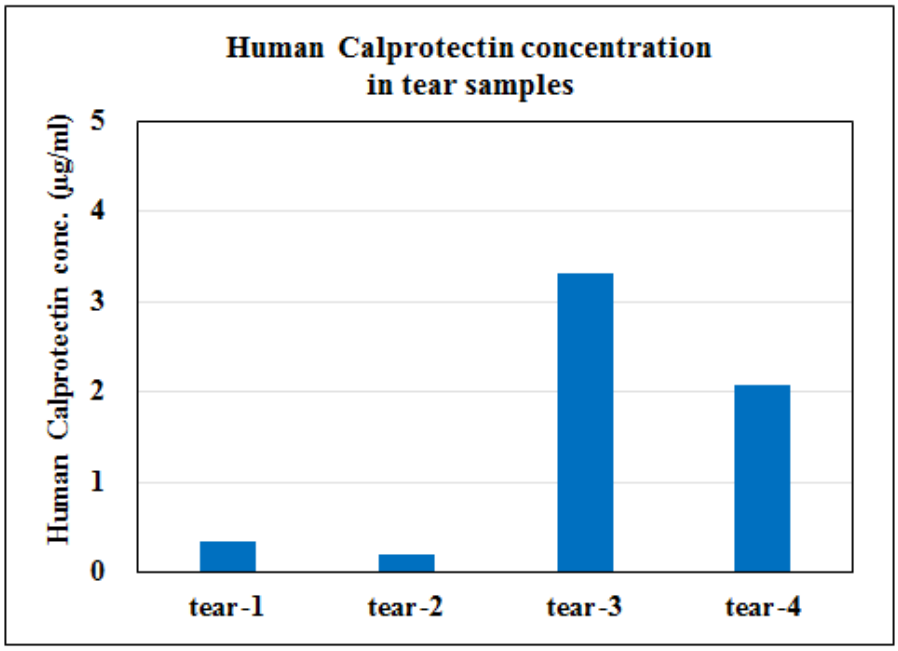


Fig.4 Human calprotectin concentration in milk samples from healthy volunteers

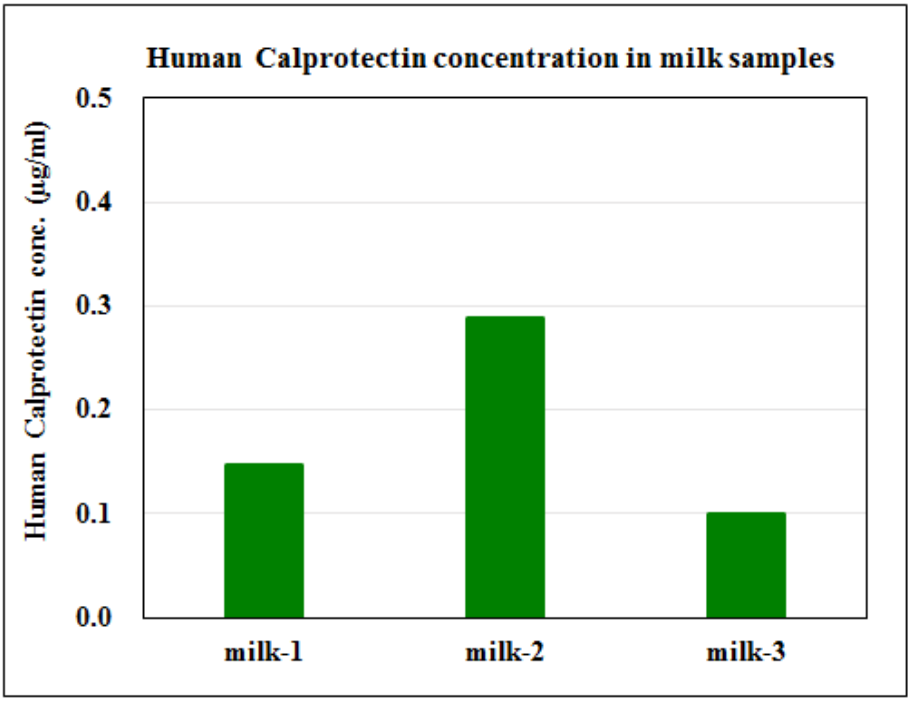


Fig.5 Human calprotectin concentration in saliva samples from healthy volunteers

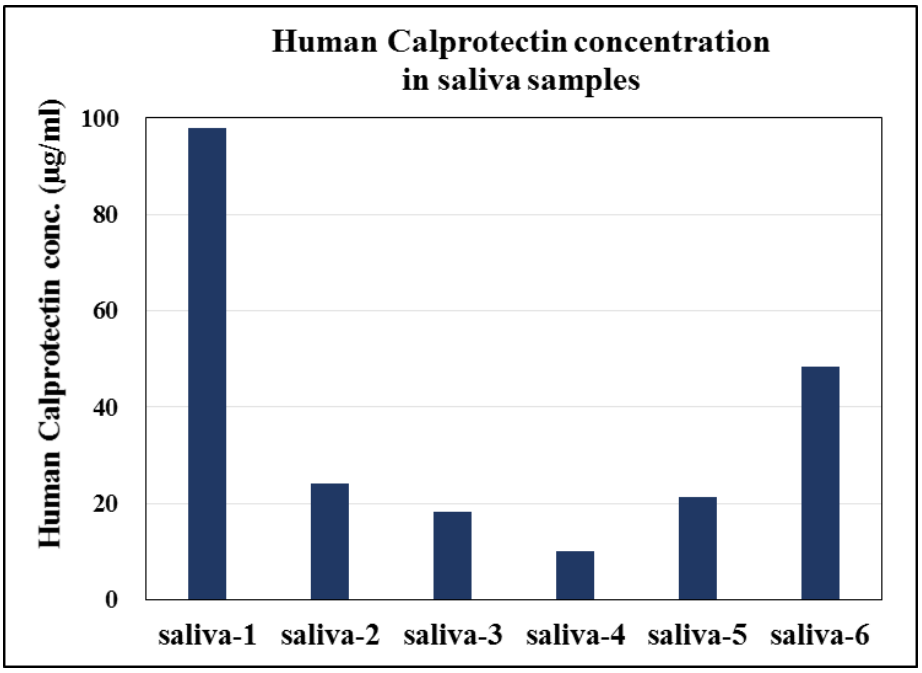


Fig.6 Human calprotectin concentration in sweat samples from healthy volunteers

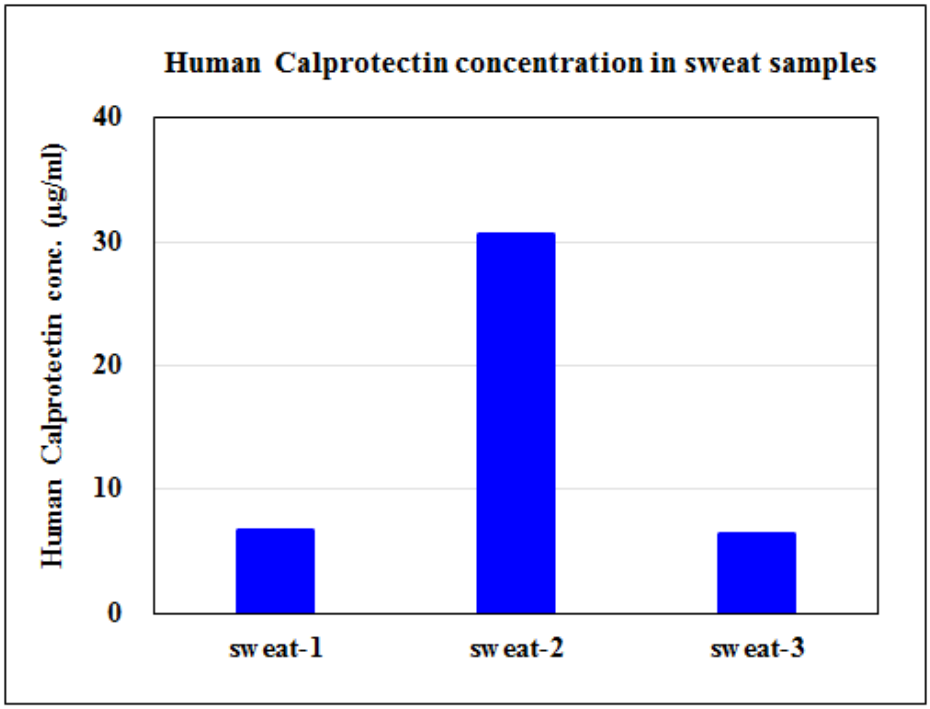
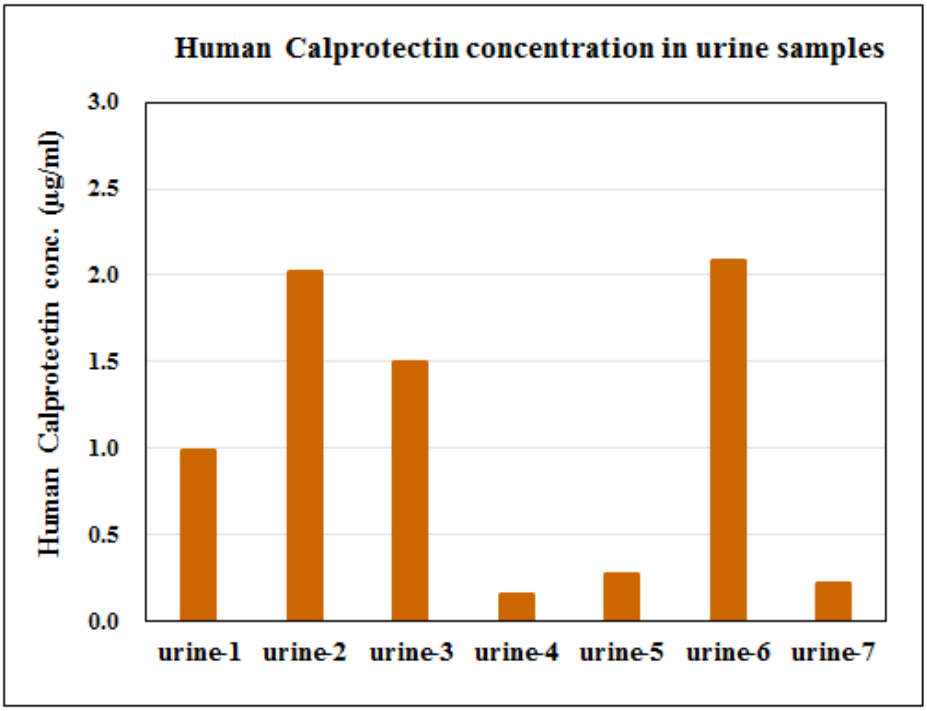


Fig.7 Human calprotectin concentration in urine samples from healthy volunteers



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