Human Calprotectin ELISA Kit

Enzyme Immunoassay for the quantification of Human Calprotectin (S100A8/S100A9 complex, a neutrophil cytoplasmic protein) in stool.

Catalog number: ARG80869

For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION **Page**

INTRODUCTION 3

PRINCIPLE OF THE ASSAY 4

MATERIALS PROVIDED & STORAGE INFORMATION 5

MATERIALS REQUIRED BUT NOT PROVIDED 6

TECHNICAL NOTES AND PRECAUTIONS 6

SAMPLE COLLECTION & STORAGE INFORMATION 8

REAGENT PREPARATION 9

ASSAY PROCEDURE 11

EXAMPLE OF TYPICAL STANDARD VALUES 13

CALCULATION OF RESULTS 16

QUALITY ASSURANCE 18

MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: No. 22, Ln. 227, Gongyuan Rd., Hsinchu City 300, Taiwan

Phone: +886 (3) 562 1738

Fax: +886 (3) 561 3008

Email: info@arigobio.com

INTRODUCTION

Calprotectin is a complex of the mammalian proteins S100A8 and S100A9. In the presence of calcium, calprotectin is capable of sequestering the transition metals iron, manganese and zinc via chelation. This metal sequestration affords the complex antimicrobial properties. Calprotectin is the only known antimicrobial manganese sequestration protein complex. Calprotectin comprises as much as 60% of the soluble protein content of the cytosol of a neutrophil, and it is secreted by an unknown mechanism during inflammation. Faecal calprotectin has been used to detect intestinal inflammation (colitis or enteritis) and can serve as a biomarker for inflammatory bowel diseases and rheumatoid arthritis. Other names for calprotectin include MRP8-MRP14, calgranulin A and B, cystic fibrosis antigen, L1, 60BB antigen, and 27E10 antigen.

The human homologue of calprotectin is a 24 kDa dimer, and is formed by the protein monomers S100A8 (10,835 Da) and S100A9 (13,242 Da). The primary structure of calprotectin can vary between species. For instance, the mouse homologue of S100A8 is 10,295 Da, while the S100A9 homologue is 13,049 Da. Early size exclusion chromatography experiments incorrectly indicated that calprotectin had a molecular mass of 36.5 kDa; occasionally this value is used in contemporary literature. Calprotectin S100A8-S100A9 dimers can non-covalently pair with one another to form 48 kDa tetramers. [Provide by Wikipedia: Calprotectin]

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A capture antibody specific for Human calprotectin has been pre-coated onto a microtiter plate. Standards, Controls or samples are pipetted into the wells and any calprotectin present is bound by the immobilized antibody. After washing away any unbound substances, an antibody-conjugate specific for calprotectin is added to each well and incubate. After washing away any unbound substances, the TMB substrate is added to the wells and color develops in proportion to the amount of calprotectin bound in the initial step. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of 450 nm. The concentration of calprotectin in the samples is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store all other components at 2-8°C. Use the kit before expiration date.

Component	Quantity	Storage information
Antibody-coated microplate	8 X 12 strips	4°C
Standards (0, 69.5, 156, 403, 892, 1860, 5560 ng/mL)	1 vial each (lyophilized)	4°C
Control 1	1 vial (lyophilized)	4°C
Control 2	1 vial (lyophilized)	4°C
Control 3	1 vial (lyophilized)	4°C
Diluent Buffer	12 mL	4°C
Antibody Conjugate (Anti-Human Calprotectin Antibody)	0.6 mL	4°C
Antibody Conjugate Diluent	12 mL (ready to use)	4°C
30X Wash Buffer	30 mL	4°C
5X Extraction Buffer	120 mL	4°C
TMB substrate	12 mL (ready to use)	4°C (protect from light)
STOP solution	12 mL (ready to use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Deionized or distilled water
- Aluminum foil
- Plastic microtiter well cover or polyethylene film
- Mixer or Ultra-Turrax
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Microtiter plate washer (recommended)

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store the kit at 2-8°C at all times.
- Prior to beginning the assay procedure, bring all reagents to room temperature (20-25°C).
- For sample values reading greater than the highest calibrator, it is recommended to re-assay samples with dilution (e.g., 1:10 or 1:100 with Extraction Buffer).
- Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.
- Avoid air bubbles in the wells as this could result in lower binding efficiency and higher CV% of duplicate reading.

- Briefly spin down the all vials before use.
- If crystals are observed in the 30X Wash Buffer, warm to 37°C until the crystals are completely dissolved.
- Minimize lag time between wash steps to ensure the plate does not become completely dry during the assay.
- Ensure complete reconstitution and dilution of reagents prior to use.
- Take care not to contaminate the TMB Substrate. Do not expose the TMB solution to glass, foil or metal. If the solution is blue before use, DO NOT USE IT.
- Do NOT return leftover TMB Substrate to bottle. Do NOT contaminate the unused TMB Substrate. If the solution is blue before use, DO NOT USE IT.
- Change pipette tips between the addition of different reagent or samples.
- Use a new adhesive plate cover for each incubation step.
- Taping the well strips together with lab tape can be done as an extra precaution to avoid plate strips from coming loose during the procedure.
- Include a standard curve each time the assay is performed.
- Run both standards and samples in at least duplicates (triplicate is recommended).

SAMPLE COLLECTION & STORAGE INFORMATION

The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design.

1. The collected fecal sample may be transported at 2-8 °C and tested within 3 days. Fecal sample may be stored below -20°C for a longer storage period. Avoid more than three freeze/thaw cycles for each specimen. Before measuring for fecal Calprotectin, vortex to dissolve stool sample.
2. The collected sample should be diluted in two steps with 1:40 and 1:9 before measurement. Following is a detailed sample extraction process.
    A. Label and tare an empty polypropylene tube together with an inoculation loop.
    B. Weigh 50-100 mg of stool using the inoculation loop by placing it into the pre-tarred tube.
    C. Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube.
    D. Add Extraction Buffer (39 parts of the stool volume, 1 g stool = 1 ml) into the tube:

Fecal Sample Weight (mg)	Extraction Buffer Volume (mL)
50	2.0
55	2.2
60	2.4
65	2.6
70	2.8
75	3.0
80	3.2
85	3.4
90	3.6

95	3.8
100	4.0

E. Vortex to dissolve stool sample. Let the sample set at room temperature vertically for 30 min for sedimentation or centrifuge the sample at 3000 x g for 5 minutes.

F. Transfer 0.15 mL clear supernatant (no particles) to a clean tube with 1.2 ml Extraction Buffer. Mix the sample by gently vortexing. This extracted sample is ready to be measured for fecal Calprotectin.

REAGENT PREPARATION

- **1X Wash Buffer**: Dilute 30X Wash Buffer into distilled water to yield 1X Wash Buffer. (E.g., add 16.7 ml of 10X Wash Buffer into 433.3 ml of distilled water to a final volume of 500 ml) The 1X Wash Buffer is stable for up to 4 weeks at 2-8°C.
- **1X Extraction Buffer**: Dilute 5X Extraction Buffer into distilled water to yield 1X Extraction Buffer. (E.g., add 10 ml of 5X Extraction Buffer into 40 ml of distilled water to a final volume of 50 ml) The 1X Extraction Buffer may be stored at room temperature and is stable until the expiration date.
- **Standards and Controls:** Reconstitute all Standards and Controls by adding 0.5 mL of distilled water to each vial. Allow the Standards and Controls to sit undisturbed for 5 minutes, and then mix well by inversions or gentle vortexing. One must make sure that all solid is dissolved completely prior to use. These reconstituted Standards and Controls may be stored at 2-8 °C for up to 3 days or at -20 °C or below for long-term storage. Do not exceed 3 freeze-thaw cycles.

- **Antibody conjugate:** Prepare Antibody Conjugate working solution by 1:21 fold dilution of the Antibody Conjugate into the Antibody Conjugate Diluent. Following is a table that outlines the relationship of strips used and antibody mixture prepared.

Strip No.	Antibody Conjugate Diluent (mL)	Antibody Conjugate (μL)
1	1	50
2	2	100
3	3	150
4	4	200
5	5	250
6	6	300
7	7	350
8	8	400
9	9	450
10	10	500
11	11	550
12	12	600

Note: This antibody working solution should be freshly prepared just before pipetting the pre-dilute Antibody Conjugate to the washed wells.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT, 20-25°C) before use. Standards and samples should be assayed in duplicates. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.

1. Add **50 µL** of **Diluent Buffer** into the microwells. Gently tap the plate to coat the well evenly.
2. Add **50 µL** of **samples, Controls and Standards** to the Antibody-coated microplate.
3. Incubate at **RT** for **1 hour** on a microplate shaker (small orbit radius **400-450 rpm**, large orbit radius, e.g. >1 cm, **150-200 rpm**).
4. Aspirate each well and wash, repeating the process 4 times for a total 5 washes. Wash by filling each well with **1× Wash Buffer (350 µL)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
5. Add **100 µL** of the **diluted Antibody Conjugate** to each well and cover with aluminum foil to protect from light.
6. Incubate at **RT** for **45 mins** on a microplate shaker (small orbit radius **400-450 rpm**, large orbit radius, e.g. >1 cm, **150-200 rpm**).
7. Aspirate each well and **wash as step 4**.
8. Warm **TMB substrate** to **RT**. Add **100 µL** of **TMB Substrate** to each well, including the blank wells. Incubate for **12 minutes (Optional 8-15 minutes)** at room temperature in the dark.

9. Read the OD with a microplate reader at **620 nm** immediately.

   Note: please shake the plate to reach a homogenous blue color distribution in the well right before reading.

10. Immediately Add **100 µL** of **Stop Solution** to each well, including the blank wells. The color of the solution should change from blue to yellow.

11. Read the OD with a microplate reader at **450 nm** immediately. (optional: read at 620 nm as reference wavelength) It is recommended reading the absorbance **within 30 minutes** after adding the stop solution.

EXAMPLE OF TYPICAL STANDARD VALUES

The following figures demonstrate typical results with the Human Calprotectin ELISA Kit. One should use the data below for reference only. This data should not be used to interpret actual results.

Note:

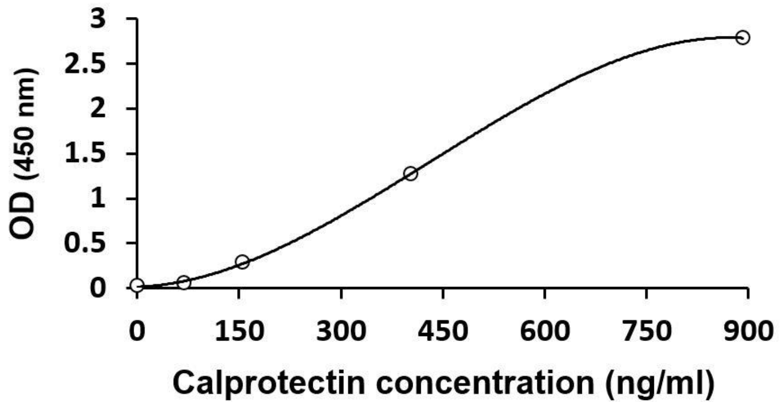
A. The fecal human calprotectin concentrations for the controls and the patient samples are read directly from the standard curve using their respective corrected absorbance.

B. Subtract the average absorbance of the Standards (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.

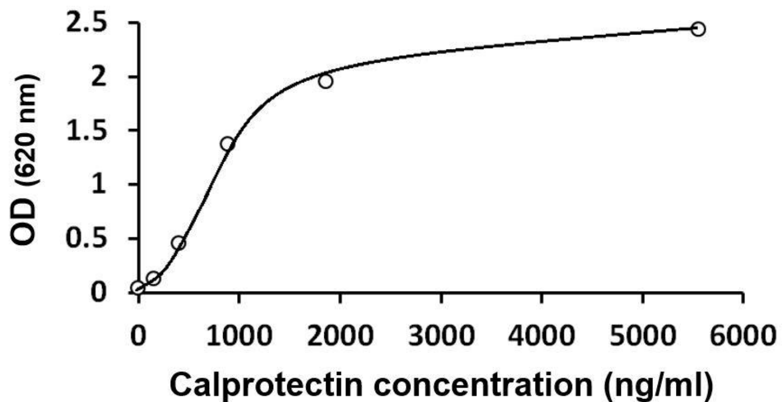
C. The standard curve is generated by the corrected absorbance of all Standards.

D. The use of the two absorbance wavelength at A620 nm and A450 nm allows for two ways to calculate sample results. It is recommended to get sample results by using the primary standard curve at A450 nm for samples with value below standard (1860 ng/mL). For samples with Calprotectin value above standard (1860 ng/mL), it is recommended to use the secondary standard curve at A620 nm.

Wells I.D.	O.D. 450 nm Absorbance			Result
	Reading	Average	Corrected	
Standard (0 ng/mL)	0.026 0.027	0.027	0.000	
Standard (69.5 ng/mL)	0.061 0.058	0.059	0.032	
Standard (156 ng/mL)	0.305 0.279	0.292	0.265	
Standard (403 ng/mL)	1.388 1.156	1.272	1.245	
Standard (892 ng/mL)	2.760 2.802	2.781	2.754	
Control 1	0.148 0.121	0.134	0.107	36.1 µg/g (100 ng/mL)
Control 2	2.601 2.614	2.607	2.580	291.4 µg/g (810 ng/mL)



Wells I.D.	O.D. 620 nm Absorbance			Result
	Reading	Average	Corrected	
Standard (0 ng/mL)	0.043 0.041	0.042	0.000	
Standard (156 ng/mL)	0.132 0.120	0.126	0.084	
Standard (403 ng/mL)	0.494 0.420	0.457	0.415	
Standard (892 ng/mL)	1.368 1.380	1.374	1.332	
Standard (1860 ng/mL)	1.945 1.950	1.948	1.906	
Standard (5560 ng/mL)	2.415 2.448	2.432	2.390	
Control 2	1.145 1.149	1.147	1.105	266.3 µg/g (740 ng/mL)
Control 3	1.778 1.779	1.779	1.737	423.1 µg/g (1176 ng/mL)



CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards and samples.
2. Stool samples from normal healthy adults with age of 24-58 were collected and measured with this ELISA. The recommended normal cut-off for fecal Calprotectin concentration by using this ELISA and sample collection system is 120 ng/mL or 43.2 µg/g directly read from assay standard curve.
   Note: Calprotectin ng/mL X 0.36 = Calprotectin µg/g
   Calprotectin µg/g X 2.78 = Calprotectin ng/mL
3. We strongly recommend that each clinical laboratory to establish its own normal cut-off level by measuring normal stool samples with this ELISA and sample collection system.
4. Please be aware that patients with recent diarrhea would give a much higher level of fecal Calprotectin. Taking spicy food or alcohol may also cause intestinal irritation resulting in an abnormal fecal Calprotectin level.
5. A strong positive of fecal calprotectin is likely to indicate a more significant clinical pathological condition of a patient. However, a low positive of fecal calprotectin does not indicate a lesser possibility of inflammation.
6. A normal fecal calprotectin level does not rule out the presence of any gastrointestinal diseases such as IBD.
7. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
8. Use the mean absorbance value for each sample determine the

corresponding concentration from the standard curve.

9. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.
10. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for the detail. (https://www.arigobio.com/elisa-analysis)
11. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

QUALITY ASSURANCE

Sensitivity

The limit of detection (LOD) of the human calprotectin is 2.5 ng/mL.

Intra-assay and Inter-assay precision

The CV value of intra-assay was 2.5-3.5 % and inter-assay precision was 2-8.6 %

Recovery

Three fecal extracts and three assay calibrators were spiked together in various volume combinations and tested. The results Calprotectin concentration in the value of ng/mL are as follows:

#	Orig. Value	Amount Spiked	Observed Value	Expected Value	Recovery %
1	30.0	37.1	61.9	67.1	92.2
2	73.0	12.7	85.7	89.3	104.2
3	217.7	30.3	248	256.9	96.5