Calprotectin ELISA Assay Kit

Catalog Number: CAL35-K01 (1 x 96 wells)

For Research Use Only. Not for use in diagnostic procedures.

v. 8.4 (22 APR 24)

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INTENDED USE

The Eagle Biosciences Calprotectin ELISA Assay kit is intended for use in the quantitative determination of human calprotectin (neutrophil cytoplasmic protein S100A8/A9) levels in stool samples via Enzyme-linked immunosorbent assay. The test is useful for detecting inflammatory bowel disease (IBD) such as ulcerative colitis and Crohn's disease. The Calprotectin ELISA Assay kit is for research use only and should not be used in diagnostic procedures.

For further information about this kit, its application, or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc. at www.EagleBio.com or at 866-411-8023.

INTRODUCTION

Quantitative determination of fecal calprotectin is an indication of the severity of bowel inflammation. Also, higher levels of calprotectin in the stool are associated with an increased risk of relapse in patients with inflammatory bowel disease (IBD). Low stool calprotectin levels correlate well with a low risk for intestinal allograft rejection. This assay uses specific monoclonal antibodies to ensure only calprotectin is detected.

ASSAY PRINCIPLE

This Calprotectin ELISA Assay Kit is designed, developed and produced for the quantitative measurement of human calprotectin in stool samples. The Calprotectin ELISA Assay Kit utilizes the two-site "sandwich" technique with two selected antibodies that bind to different epitopes of human calprotectin.

Assay standards, controls and patient samples are added directly to wells of a microtiter plate that is coated with antibody to calprotectin. After a short incubation period, the plate is washed and horseradish peroxidase (HRP) conjugated human calprotectin specific monoclonal antibody is added to each well. After the second incubation period, a "sandwich" of solid-phase antibody - human calprotectin – HRP conjugated monoclonal antibody" is formed. The unbound monoclonal antibodies and buffer matrix are removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to the wall of each microtiter well is directly proportional to the amount of human calprotectin in the test sample. A standard curve is generated by plotting the absorbance versus the respective human calprotectin concentration for each standard on a point-to-point or 4-parameter curve fitting. The concentration of fecal human calprotectin in test samples is determined directly from this standard curve of the Calprotectin ELISA Assay.

PREPARATION AND STORAGE

1. **Calprotectin Antibody Coated Microplate**
   One ready to use Microplate (1 x 96 wells) coated with Anti-Calprotectin antibody. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the Calprotectin ELISA Assay Kit box.

2. **Calprotectin Tracer Antibody**

   One vial containing 0.6mL HRP labeled anti-human calprotectin antibody stabilized protein matrix This reagent must be diluted with Tracer Antibody Diluent before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the Calprotectin ELISA Assay Kit box

3. **Elisa Wash Concentrate**

   One bottle contains 30 mL of 30-fold concentrate. Before use the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non- mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the Calprotectin ELISA Assay Kit box.

4. **ELISA HRP Substrate**

   One bottle contains 12 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the Calprotectin ELISA Assay Kit box.

5. **ELISA Stop Solution**

   One bottle contains 12 mL of 2N Hydrochloric Acid (HCl). This reagent may be stored at 2 – 8°C or room temperature and is stable until the expiration date on the Calprotectin ELISA Assay Kit box. **Caution: this component contains potentially hazardous material.**

6. **Calprotectin Standards**

   Seven vials containing human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative**. Refer to vials for exact concentration for each standard.** These reagents should be stored at 2 – 8°C and are stable until the expiration date on the Calprotectin ELISA Assay Kit box

7. **Calprotectin Controls**

   Three vials containing human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. **Refer to vials for exact concentration range for each control.** Both controls should be stored at 2 – 8 °C and are stable until the expiration date on the Calprotectin ELISA Assay Kit box

8. **Tracer Antibody Diluent**

   One bottle containing 12 mL ready to use buffer. It should be used according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the Calprotectin ELISA Assay Kit box.

9. **Assay Buffer**

   One bottle containing 12 mL ready to use buffer. It should be used according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the Calprotectin ELISA Assay Kit box

10. **Extraction Buffer Concentrate**

    One bottle containing 120 mL of 5-fold concentrate. Before use the contents must be diluted with **480 mL** of demineralized water and

mixed well. Upon dilution, this yields a ready-to-use Extraction Buffer for fecal sample extraction and dilution. The diluted Extraction Buffer may be stored at room temperature and is stable until the expiration date on the Calprotectin ELISA Assay Kit box.

SAFETY PRECAUTIONS

The reagents of the Calprotectin ELISA Assay Kit must be used in a research setting. The source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Hydrochloric acid may cause severe irritation on contact with skin. Provide good ventilation in process area to prevent formation of vapor. Do not breath mist, vapors, spray. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Fecal sample collection tube (Catalog Number CAL35-C50)
2. Precision single channel pipettes capable of delivering 50 µL, 100 µL, 500 µL, etc.
3. Disposable pipette tips suitable for above volume dispensing.
4. Disposable plastic 100 mL and 1000 mL bottle with caps.
5. Aluminum foil.
6. Deionized or distilled water.
7. Plastic microtiter well cover or polyethylene film.
8. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
9. Spectrophotometric microplate reader capable of reading absorbance at 450 nm and 650 or 630

SPECIMEN COLLECTION

1. Only one fecal sample is required. Fresh fecal sample must be collected by using Eagle Biosciences Fecal Sample Collection Tube (Cat. No. CAL35-C50). This tube is specially designed for easy collection of a substantially small amount of fecal sample into the tube pre-filled with sample extraction buffer. The collected fecal sample may be transported at ambient temperature, stored 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 times freeze - thaw cycle for each specimen. Before measuring for fecal Calprotectin, vortex to dissolve stool sample.

Note: The validation data of this Calprotectin ELISA was generated by using Fecal Sample Collection Tube. To order this tube, please order Fecal Calprotectin Sample Collection kit (Cat. No. CAL35-C50) and each kit contains 50 tubes filled with extraction buffer. A different calprotectin test result may be obtained by using a different type of fecal sample collection tube.

2. It is an alternative to collect fecal sample with a commercial stool sample collection device. The collected sample can be stored at 2-8 °C for up to 6 days. 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) Weight 50 – 100 mg of stool using the inoculation loop by placing it into the pre-tared tube.
c) Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube
d) For every 1 volume of the stool, as 49 volume of Extraction buffer into the tube (stool volume calculation: 100 mg stool = 100 µL of stool). The following is an easy to use reference extraction procedure.

Fecal Sample Weight (mg)	Extraction Buffer Volume (ml)
50 -54	2.0
55 - 59	2.2
60 - 64	2.4
65 - 69	2.6
70 - 74	2.8
75 - 79	3.0
80 - 84	3.2
85 - 89	3.4
90 - 94	3.6
95 - 99	3.8
100 - 104	4.0

e) Vortex to dissolve stool sample. Let the sample set at room temperature vertically for 30 for sedimentation or centrifuge the sample at 3000 x g for 5 minutes.
f) Transfer 0.15 mL clear supernatant (no particles) to clean tube with 1.2 mL Extraction Buffer. Mix samples by gently vortexing. This extracted sample is ready to be measured for fecal Calprotectin.

ASSAY PROCEDURE

1. **Reagent Preparation**
    1. Prior to use allow all reagents of the Calprotectin ELISA Assay Kit to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
    2. ELISA Wash Concentrate must be diluted to working solution prior to use. Please see REAGENTS section for details.
    3. Reconstitute all assay standard level 1 to level 7 and controls by adding **0.5 mL** of demineralized 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 –10 °C or below for long-term storage. Do not exceed 3 freeze-thaw cycles
    4. Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	C 2
B	STD 1	STD 5	C 2
C	STD 2	STD 6	C 3
D	STD 2	STD 6	C 3
E	STD 3	STD 7	SAMPLE 1
F	STD 3	STD 7	SAMPLE 2
G	STD 4	C 1	SAMPLE 3
H	STD 4	C 1	SAMPLE 4

    5. Place a sufficient number of calprotectin coated microwell strips in a holder to run human calprotectin standards, controls and unknown samples in duplicate.
    6. Prepare Tracer Antibody working solution by 1:21 fold dilution of the Calprotectin Tracer Antibody by adding the tracer antibody into the Tracer Antibody Diluent. Following is a table that outlines the relationship of strips used and antibody mixture prepared

Strip no.	Tracer Antibody Diluent	Tracer Antibody
1	1 mL	50 µL
2	2 mL	100 µL
3	3 mL	150 µL
4	4 mL	200 µL
5	5 mL	250 µL
6	6 mL	300 µL
7	7 mL	350 µL
8	8 mL	400 µL
9	9 mL	450 µL

10	10 mL	500 µL
11	11 mL	550 µL
12	12 mL	600 µL

Note: this antibody working solution should be freshly prepared just before pipetting the tracer antibody to the washed wells.

2. **Patient Sample Preparation**
    1. If the Eagle Biosciences Fecal Sample Collection Tube is used, there is no sample preparation required.
3. **Assay Procedure**
    1. Add **50 µL** of Assay Buffer into the designated microwells. Gently tap the plate to coat the wells evenly.
    2. Add **50 µL** of Standards, Controls and extracted patient samples into the designated microwells
    3. Seal the plate wells securely, cover with foil or other material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for 1 hr. ± 5 minutes at 400 to 450 rpm.
    4. Just prior to the end of the incubation time, dilute the proper amount of Tracer Antibody for the assay.
    5. **Wash** each well 5 times by dispensing **350 µL** of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
    6. Add **100 µL** of above Tracer Antibody to each well.
    7. Seal the plate wells securely, cover with foil or other material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for 45 minutes ± 5 minutes at 400 to 450 rpm.
    8. Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
    9. Add **100 µL** of ELISA HRP Substrate into each of the wells.
    10. Cover the plate with aluminum foil to or other material to avoid exposure to light. Incubate plate static, at room temperature, for **12 minutes** (Optional 8 - 15 minutes).
    11. Remove the aluminum foil. Read the absorbance at **620 nm** (optional wavelengths from 595 nm to 650 nm depending on available filters) **immediately**. Note: please shake the plate to reach a homogenous blue color distribution in the well right before reading.
    12. Immediately add **100 µL** of ELISA Stop Solution into each of the wells. Mix gently.
    13. Read the absorbance at 450 nm with reference filter at 620 nm or 650 nm.

Procedural Notes

1. It is recommended that all standards, controls and unknown samples be assayed in duplicate with the Calprotectin ELISA Assay Kit. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results. Keep light sensitive reagents in the original amber bottles.
2. Keep light sensitive reagents in the original amber bottles.
3. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. An orbital mixer with a larger orbit radius (e.g. > 1 cm) may be used at speeds of 150 to 200 rpm
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents of the Calprotectin ELISA Assay Kit should be mixed gently and thoroughly prior to use. Avoid foaming.
9. If adapting this assay to automated ELISA system such as DS-2, a procedural validation is necessary if there is any modification of the assay procedure.

INTERPRETATION OF RESULTS

It is recommended to use a point-to-point or 4-parameter standard curve fitting.

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the level 1 standard (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.

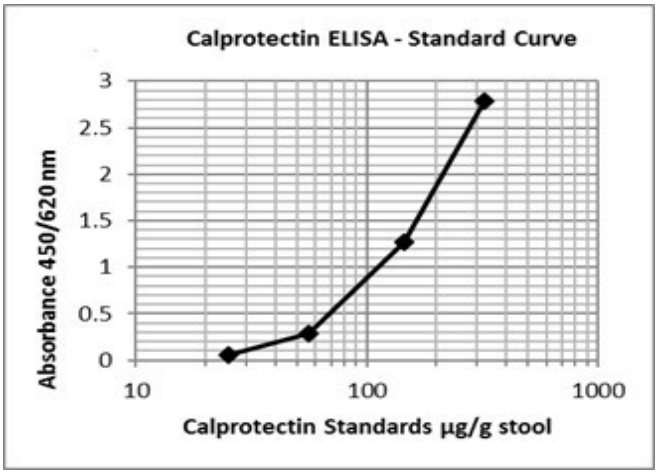
The fecal human calprotectin concentrations for the controls and the patient samples are read directly from the standard curve using their respective corrected absorbance.

The use of the two absorbance wavelength at A 620 nm and A450/620 nm allows for two ways to calculate sample results. It is recommended to read sample results by using the primary standard curve at A 450/620 nm for samples with value below standard level 5. While for samples Calprotectin value above standard level 5, it is recommend using the secondary standard curve at A 620 nm.

EXAMPLE DATA AND STANDARD CURVE (Low)

A typical absorbance data and the resulting standard curve from this fecal human calprotectin ELISA are represented**. This curve should not be used in lieu of standard curve run with each assay.**

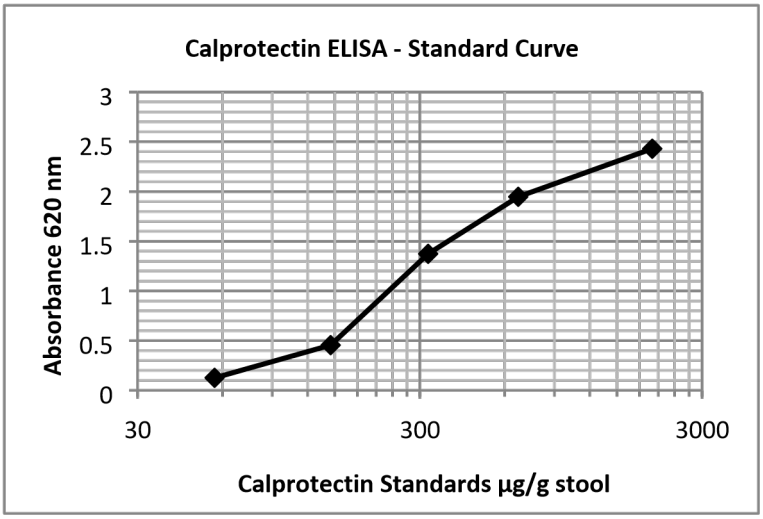
Well I.D.	OD 450 nm Absorbance			Results
	Readings	Average	Corrected	
Std-1: 0 µg/g (0 ng/mL)	0.026 0.027	0.027	0.000	
Std-2: 25 µg/g (69.5 ng/mL)	0.061 0.058	0.059	0.032	
Std-3: 56.2 µg/g (156 ng/mL)	0.305 0.279	0.292	0.265	
Std-4: 145 µg/g (403 ng/mL)	1.388 1.156	1.272	1.245	
Std-5: 321 µg/g (892 ng/mL)	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)



EXAMPLE DATA AND STANDARD CURVE (High)

A typical absorbance data and the resulting standard curve from this fecal human calprotectin ELISA are represented. **This curve should not be used in lieu of standard curve run with each assay.**

Well I.D.	OD 620 nm Absorbance			Results
	Readings	Average	Corrected	
Std-1: 0 µg/g (0 ng/mL)	0.043 0.041	0.042	0.000	
Std-3: 56.2 µg/g (156 ng/mL)	0.132 0.120	0.126	0.084	
Std-4: 145 µg/g (403 ng/mL)	0.494 0.420	0.457	0.415	
Std-5: 321 µg/g (892 ng/mL)	1.368 1.380	1.374	1.332	
Std-6: 669 µg/g (1860 ng/mL)	1.945 1.950	1.948	1.906	
Std-7: 2000 µg/g (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 µg/g (1176 ng/ml)



EXPECTED VALUES

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.** 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.

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.

Note: Calprotectin ng/mL X 0.36 =

Calprotectin µg/g

Calprotectin µg/g X 2.78 =

Calprotectin ng/mL

Please program ELISA reader by selecting assay standards concentration either in "µg/g" or
"ng/mL to avoid manual calculation!

LIMITATION OF PROCEDURE

1. 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.
2. A normal fecal calprotectin level does not rule out the presence of any gastrointestinal diseases such as IBD.
3. For sample values reading greater than the highest standard, it is recommended to re-assay samples with dilution (i.e. 1:10 or 1:100 with Extraction Buffer).
4. Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

PERFORMANCE CHARACTERISTICS

Sensitivity

The analytical sensitivity (LLOD) of the human Calprotectin ELISA Assay Kit as determined by the 95% confidence limit on 12 duplicate determination of zero standard is approximately 2.5ng/mL. A LLOQ was determined by dilution of assay standards and it is about 5 ng/mL.

High Dose "hook" effect

This assay has showed that it did not have any high dose "hook" for calprotectin level up to 40,000 ng/mL in extraction buffer

PRECISION

Intra-assay

intra-assay precision was validated by measuring three sample extracts in a single assay with 12 replicate determinations

Mean Calprotectin Value (µg/g)	CV (%)
5.74	2.9
26.59	3.5
54.70	2.5

Inter-assay

The inter-assay precision was validated by measuring two samples in duplicate in 4 individual assays.

Mean Calprotectin Value (µg/g)	CV (%)
21.64	8.6
70.31	2.0

The precision of inter-sample collection was performed by collecting five specimens from one bowel movement. These grouped samples are measured in an assay according to the assay procedure. The results of Calprotectin concentration in the value of ng/mL indicate that there are very satisfactory agreements of the five samples collected from one bowel movement.

Donor	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	CV%
A	57.0	65.0	59.2	56.2	49.8	9.5
B	60.4	55.3	58.8	71.7	81.1	16.3
C	72.3	69.3	51.5	65.7	65.6	12.3

Linearity

One sample was diluted with assay buffer and tested. The results of Calprotectin concentration in the value of ng/mL are as follows:

DILUTION	OBSERVED VALUE	EXPECTED VALUE	RECOVERY %
Neat	195.84	-	-
1:2	87.88	97.92	89.7
1:4	46.58	48.96	95.1
1:8	24.53	24.48	100.2
1:16	13.77	12.24	112.5

Spike Recovery

Three fecal extracts and three assay standards 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	248.0	256.9	96.5

Calprotectin ELISA: Condensed Assay Protocol

1. 50 µL Assay Buffer per well

2. 50 µL Calibrators, controls and extracted patient samples
   Incubate @ RT for 60 min on ELISA plate shaker Wash 5 x

3. 100 µL Tracer Antibody
   Incubate @ RT for 45 min on ELISA plate shaker Wash 5 x

4. 100 µl TMB Substrate
   Incubate @ RT for 12 min static

5. Read absorbance at 620 nm Immediately

6. 100 µl Stop Solution

7. Read absorbance at 450/620 or 450/650 n

REFERENCES

1. Tibble et al. A simple method for assessing intestinal inflammation in Crohn's disease. Gut.2000;47:506-513
2. Costa F et al. Calprotectin is a stronger predictive marker of relapse in ulcerative colitis than in Crohn's disease. Gut. 2005;54:364-8

Warranty Information

Eagle Biosciences, Inc. warrants its Product(s) to operate or perform substantially in conformance with its specifications, as set forth in the accompanying package insert. This warranty is expressly limited to the refund of the price of any defective Product or the replacement of any defective Product with new Product. This warranty applies only when the Buyer gives written notice to the Eagle Biosciences within the expiration period of the Product(s) by the Buyer. In addition, Eagle Biosciences has no obligation to replace Product(s) as result of a) Buyer negligence, fault, or misuse, b) improper use, c) improper storage and handling, d) intentional damage, or e) event of force majeure, acts of God, or accident.

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