Calprotectin ELISA Assay Kit

Catalog Number:
CAL35-K01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
v. 21 (effective 04APR23)

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INTENDED USE

The Eagle Biosciences Calprotectin ELISA Assay Kit (enzyme-linked immunoassay kit) is intended for the quantitative determination of human calprotectin (neutrophil cytoplasmic protein S100A8/A9) levels in stool samples. The Eagle Biosciences Calprotectin ELISA Assay Kit is for research use only and not to 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.

PRINCIPLE OF THE ASSAY

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 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.

LIMITATIONS RELATED TO INTENDED USE

- A strong positive of fecal calprotectin is likely to indicate a more significant pathological condition of a sample. However, a low positive of fecal calprotectin does not indicate a lesser possibility of inflammation.
- A normal fecal calprotectin level does not rule out the presence of any gastrointestinal diseases such as IBD.
- 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).
- Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

PROCEDURAL WARNINGS AND PRECAUTIONS

- This kit is for use by trained laboratory personnel (professional use only). For research use only.
- Practice good laboratory practices when handling kit reagents and specimens. This includes:
- Do not pipette by mouth.
- Do not smoke, drink, or eat in areas where specimens or kit reagents are handled.
- Wear protective clothing and disposable gloves.

- Wash hands thoroughly after performing the test.
- Avoid contact with eyes, use safety glasses; in case of contact with eyes, flush eyes with water immediately and contact a doctor.
- Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
- Do not use this kit beyond the expiry date stated on the label.
- If the kit reagents are visibly damaged, do not use the test kit.
- Do not use kit components from different kit lots within a test and do not use any component beyond the expiration date printed on the label.
- All kit reagents and specimens must be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of specimens.
- When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
- Immediately after use, each individual component of the kit must be returned to the recommended storage temperature stated on the label.
- A calibrator curve must be established for every run.
- It is recommended to all customers to prepare their own control materials or sample pools which should be included in every run at a high and low level for assessing the reliability of results.
- The controls (if applicable with this kit) must be included in every run and their results must fall within the ranges stated in the quality control certificate; a failed control result might indicate improper reagent storage.
- When dispensing the substrate and stopping solutions, do not use pipettes in which these liquids will come into contact with any metal parts.
- The TMB Substrate is sensitive to light and should remain colorless if properly stored. Instability or contamination may be indicated by the development of a blue color, in which case it should not be used.
- Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
- Samples or controls containing azide or thimerosal are not compatible with this kit, they may lead to false results.
- Avoid microbial contamination of reagents.
- To prevent the contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, calibrator, and control.
- To prevent contamination of reagents, do not pour reagents back into the original containers.
- Kit reagents must be regarded as hazardous waste and disposed of according to local and/or national regulations.
- Consumables used with the kit that are potentially biohazardous (e.g., pipette tips, bottles or containers containing human materials) must be handled according to biosafety practices to minimize the risk of infection and disposed of according to local and/or national regulations relating to biohazardous waste.
- This kit contains 2N Hydrochloric Acid in the stopping solution component. Do not combine acid with waste material containing sodium azide or sodium hypochlorite.
- The use of safety glasses, and disposable plastic, is strongly recommended when manipulating biohazardous or bio-contaminated solutions.
- Proper calibration of the equipment used with the test, such as the pipettes and absorbance microplate reader, is required.
- If a microplate shaker is required for the assay procedure, the type and speed of shaker required is stated in the REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED section. Both the type and speed of shaker used can influence the optical densities and test results. If a different type of shake and/or speed is used, the user is responsible for validating the performance of the kit.

- Do not reuse the microplate wells, they are for SINGLE USE only.
- To avoid condensation within the microplate wells in humid environments, do not open the pouch containing the microplate until it has reached room temperature.
- Any serious incident that has occurred in relation to the device shall be reported to the manufacturer and the competent authority of the Member State in which the user and/or the participant is established.
- When reading the microplate, the presence of bubbles in the wells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.

SAFETY CAUTIONS AND WARNINGS

BIOHAZARDS

The reagents should be considered a potential biohazard and handled with the same precautions applied to human specimens, All human specimens should be considered a potential biohazard and handled as if capable of transmitting infections and in accordance with good laboratory practices.

CHEMICAL HAZARDS

Avoid direct contact with any of the kit reagents. Specifically avoid contact with the TMB Substrate (contains tetramethylbenzidine) and Stopping Solution (contains sulfuric acid). If contacted with any of these reagents, wash with plenty of water and refer to SDS for additional information.

SPECIMEN COLLECTION, STORAGE, AND PRE-TREATMENT

Specimen Collection & Storage

1. Only one fecal sample is required. Fresh fecal sample may 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.

Specimen Pre-Treatment

Using Calprotectin Fecal Sample Collection Kit (CAL35-C50)

If the Calprotectin Fecal Sample Collection Kit (CAL35-C50) is used, there is no sample preparation required.

Manual Weighing

The collected sample should be diluted in two steps with 1:40 and 1:9 before measurement. Following is a detailed sample extraction process.

1. Label and tare an empty polypropylene tube together with an inoculation loop.
2. Weight 50 – 100 mg of stool using the inoculation loop by placing it into the pre-tared tube.
3. Record the net amount of sample and break the inoculation loop; leave the lower part of the loop in the tube
4. For every 1 volume of the stool, add 39 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

5. Vortex to dissolve stool sample. Let the sample set at room temperature vertically for 30 minute for sedimentation or centrifuge the sample at 3000 x g for 5 minutes.
6. 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.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

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

REAGENTS PROVIDED

1. **Calprotectin Antibody Coated Microplate**

Contents:	One 1x96 well plate coated with anti-calprotectin antibody.
Format:	Ready to Use
Storage:	2-8°C
Stability:	Stable until the expiry date printed on the label.

2. **Calprotectin Tracer Antibody (21x)**

Contents:	One bottle of HRP-labeled calprotectin antibody in a stabilized protein matrix
Format:	Concentrated; Requires Preparation
Volume:	0.6 mL/bottle
Storage:	2-8°C
Stability:	Stable until the expiry date printed on the label.
Preparation of Tracer Antibody:	**Dilute 1:21** with tracer antibody diluent and mix well before use.

3. **ELISA Wash Concentrate (30x)**

Contents:	One bottle of surfactant in a phosphate buffered saline with non-azide preservative.
Format:	Concentrated; Requires Preparation
Volume:	30 mL/bottle
Storage:	2-8°C
Stability:	Stable until the expiry date printed on the label.
Preparation of Wash Solution:	**Dilute 1:30**. Combine contents with 870 mL distilled water and mix well before use.

4. **ELISA HRP Substrate**

Contents:	One bottle of tetramethylbenzidine (TMB) with stabilized hydrogen peroxide.
Format:	Ready to Use
Volume:	12 mL/bottle
Storage:	2-8°C
Stability:	Stable until the expiry date printed on the label.

5. **ELISA Stop Solution**

Contents:	One bottle of 2N Hydrochloric Acid (HCl)
Format:	Ready to Use
Volume:	12 mL/bottle
Storage:	2-8°C
Stability:	Stable until the expiry date printed on the label.

6. **Calprotectin Standards 1 - 7**

Contents:	Seven bottles 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.
Format:	Concentrated; Requires Preparation
Storage:	2-8°C, <-20°C for long term storage. Do not exceed 3 freeze-thaw cycles.
Stability:	Unopened: Stable until the expiry date printed on the label. Following Preparation: Up to 3 days at 2-8°C or -10°C or below for long-term storage.
Preparation of Standards:	Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mix well by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

7. **Calprotectin Controls**

Contents:	Three bottles of human calprotectin in a lyophilized bovine serum-based matrix with a non-azide, non-mercury preservative. Refer to vials for exact concentrations for each standard.
Format:	Concentrated; Requires Preparation
Storage:	2-8°C, <-20°C for long term storage. Do not exceed 3 freeze-thaw cycles.
Stability	Unopened: Stable until the expiry date printed on the label. Following Preparation: Up to 3 days at 2-8°C or -10°C or below for long-term storage.
Preparation of Controls:	Must be reconstituted with 0.5 mL of demineralized water, allowed to sit for 10 minutes, and then mix microwell by inversions or gentle vortexing. Make sure that all solids are dissolved completely prior to use.

8. **Tracer Antibody Diluent**

Contents:	One bottle containing tracer antibody diluent
Format:	Ready to Use
Volume:	12 mL/bottle
Storage:	2-8°C
Stability:	Stable until expiry date printed on the label.

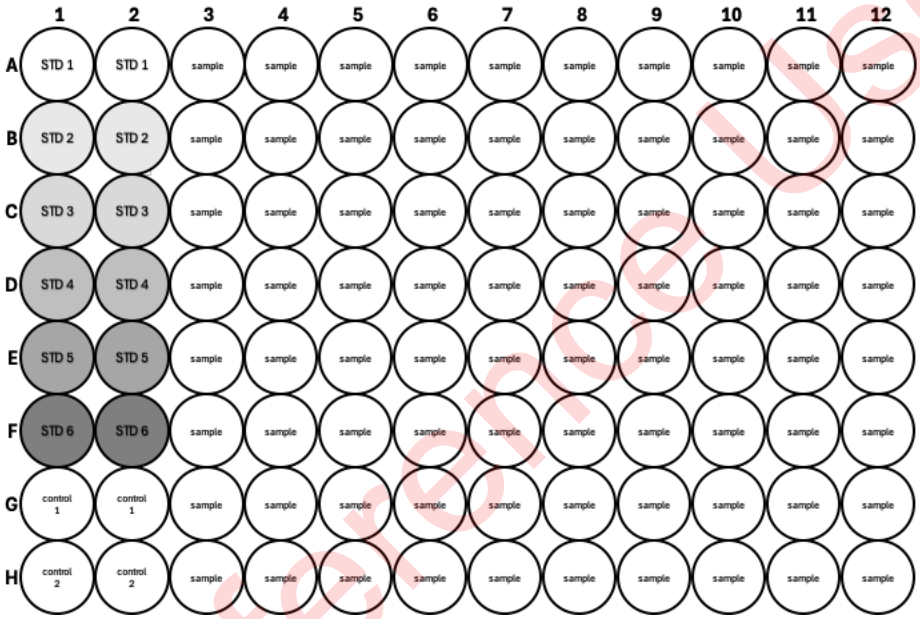
9. **Assay Buffer**

Contents:	One bottle of buffer to be used according to the assay procedures.
Format:	Ready to Use
Volume:	12 mL/bottle
Storage:	2-8°C
Stability:	Stable until expiry date printed on the label

10. **Extraction Buffer Concentrate (5x)**

Contents:	One bottle of buffer to be used according to the assay procedures.
Format:	Concentrated; Requires Preparation
Volume:	120 mL/bottle
Storage:	2-8°C
Stability:	Stable until expiry date printed on the label.
Preparation of Extraction Buffer Solution:	**Dilute 1:5** Combine contents with 480 mL of demineralized water and mixed well before use.

RECOMMENDED ASSAY LAYOUT*



**Layout subject to change based on standard and control quantities*

ASSAY PROCEDURE

All kit components, controls, and specimen samples must reach room temperature prior to use. Calibrators, controls, and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Place a sufficient number of microwell strips in a holder to run calibrators, controls, and extracted samples in duplicate.
2. Add **50 µL** of Assay Buffer into the designated microwells. Mix by gently tapping the plate.
3. Add **50 µL** of Calibrators, Controls and extracted samples into the designated microwells. Mix by gently tapping the plate.
4. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25°C)** with **shaking at 400 to 450 rpm** for **60 minutes**.

5. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
6. Prepare the antibody working solution by 1:21 fold dilution of the tracer antibody with the diluent. For each strip, it is required to mix 1 mL of the tracer antibody diluent with 50 µL of the tracer antibody in a clean test tube.
   Note: This antibody working solution should be freshly prepared.
7. Add **100 µL** of above antibody working solution to each microwell.
8. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25°C)** with **shaking at 400 to 450 rpm** for **45 minutes.**
9. Remove the plate sealer. Aspirate the contents of each microwell. Wash each well **5 times** by dispensing **350 µL** of diluted wash solution into each well, then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
10. Add **100 µL** of substrate into each microwell. Mix by gently tapping the plate.
11. Cover the plate with one plate sealer and aluminum foil. Incubate at **room temperature (20-25°C)** for **12 minutes.**
12. Remove the aluminum foil and plate sealer.
13. Read the absorbance at **620 nm** (optional wavelengths from **595** nm to **650** nm depending on available filters) **immediately** with a microplate reader.
14. Immediately add **100 µL** of Stop Solution into each of the wells. Mix by gently tapping the plate.
15. Read the absorbance at **450/620 nm** or **450/640 nm** within 10 minutes with a microplate reader.

CALCULATIONS

1. It is recommended to use a point-to-point or 4-parameter standard curve fitting.
2. Calculate the average absorbance for each pair of duplicate test results.
3. Subtract the average absorbance of the level 1 standard (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
4. 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.
5. The fecal human calprotectin concentrations for the controls and the samples are read directly from the standard curve using their respective corrected absorbance.
6. 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. For samples with calprotectin value above standard level 5, it is recommend using the secondary standard curve at A 620 nm.

QUALITY CONTROL

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

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. Consuming spicy food or alcohol may also cause intestinal irritation resulting in an abnormal fecal Calprotectin level.

Calprotectin ng/mL X 0.36 = Calprotectin µg/g

Calprotectin µg/g X 2.78 = Calprotectin ng/mL

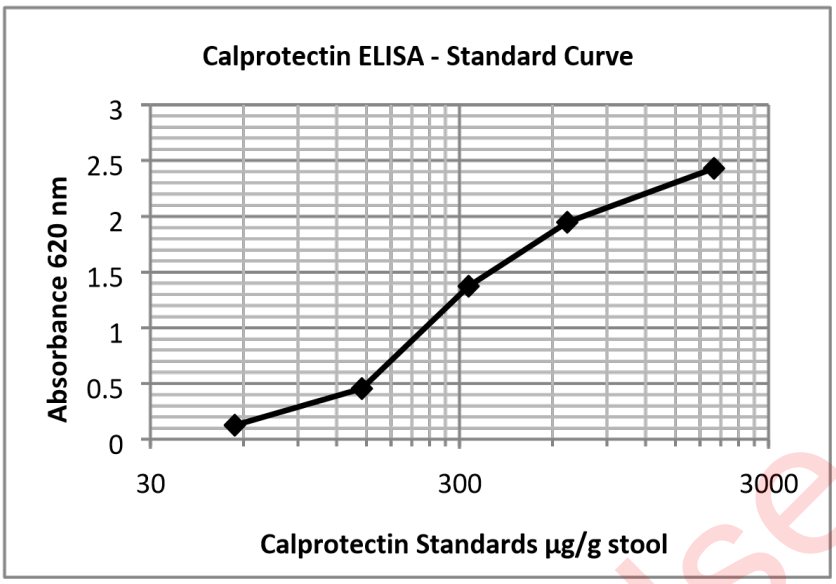
Note: Please program ELISA reader by selecting assay standards concentration either in "µg/g" or "ng/mL" to avoid manual calculation!

TYPICAL DATA

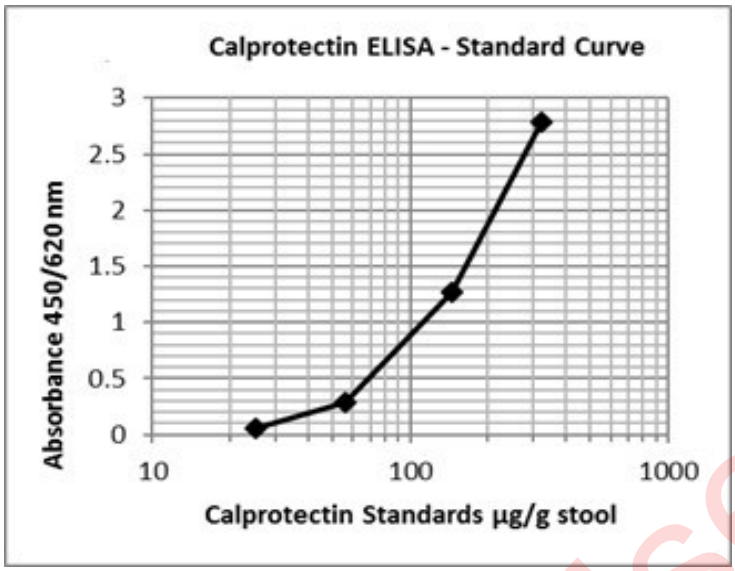
A typical absorbance data and the resulting standard curves from are represented.

Note: This curve should not be used in lieu of calibrator curve run with each assay.

Well ID	Readings (620 nm)	Average	Corrected	Concentration µg/g (ng/mL)
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 (740)
Control 3	1.778 1.779	1.779	1.737	423 (1176)



Well ID	Readings (450/620 nm)	Average	Corrected	Concentration µg/g (ng/mL)
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 1.155	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 (100)
Control 2	2.601 2.614	2.607	2.580	291.4 (810)



PERFORMANCE AND 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 2.5 ng/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.

Intra-Assay Precision

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

Sample	Mean	CV %
1	5.74	2.9
2	26.59	3.5
3	54.70	2.5

Inter-Assay Precision

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

Sample	Mean	CV %
1	21.64	8.6
2	70.31	2.0

Inter-Sample Precision

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 are as follows:

Sample	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Sample 1	195.84	-	-
50%	87.88	97.92	89.7
25%	46.58	48.96	95.1
12.5%	24.53	24.48	100.2
6.25%	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:

Sample	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Standard 1	61.9	67.1	92.2
Standard 2	85.7	89.3	104.2
Standard 3	248.0	256.9	96.5

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