BÜHLMANN

BÜHLMANN fCAL® ELISA

Calprotectin

For *In Vitro* Diagnostic Use

Rx Only

EK-CAL	96 tests
EK-CAL2	192 tests

CLIA Complexity: High

BÜHLMANN LABORATORIES AG
Baselstrasse 55
4124 Schönenbuch, Switzerland
Tel.: +41 61 487 1212
Fax: +41 61 487 1234
info@buhlmannlabs.ch

ENGLISH

INTENDED USE

The BÜHLMANN fCAL® ELISA is an in vitro diagnostic assay intended for the quantitative measurement of fecal calprotectin in human stool. The BÜHLMANN fCAL® ELISA aids in the diagnosis of inflammatory bowel disease (IBD), specifically Crohn's disease (CD) and ulcerative colitis (UC) and aids in the differentiation of IBD from irritable bowel syndrome (IBS) in conjunction with other laboratory and clinical findings

SUMMARY AND EXPLANATION OF THE TEST

Gastroenterologists are often faced with the diagnostic difficulty of differentiating individuals with functional gastrointestinal disorders, such as irritable bowel syndrome (IBS), from those with inflammatory bowel disease (IBD). Many symptoms are common to both conditions, whereas other clinical features such as a predominance of diarrhea and rectal bleeding will increase the likelihood of inflammatory disease. The clinical differentiation between these conditions remains problematic and may result in delayed diagnosis. Furthermore many individuals with IBS must undergo invasive procedures (endoscopy) to rule out an organic disorder. This has significant implications for health care costs as well as exposing individuals to the inherent risks associated with invasive procedures (ref. 1-4).

Diseases included in the IBD category include Crohn´s disease (CD), ulcerative colitis (UC) and indeterminate colitis. IBD represents chronic and often disabling lifelong inflammatory conditions – frequently diagnosed in young people in their late teens and early twenties. It is estimated that nearly 1.2 million Americans are living with IBD and those prevalence rates are rising (ref. 5). The main difference between CD and UC is the location and nature of the inflammatory condtion. In UC, the disease is restricted to the colon, wheras in CD, inflammation may affect any part of the gastrointestinal tract – the ileo-cecal area being most often affected (ref. 6, 7).

The most striking difference between IBS and IBD is that the former is non-inflammatory in nature. Therefore, one possibility is to measure surrogate markers of intestinal inflammation to differentiate between the two (ref. 8, 9). Calprotectin is a calcium-binding protein found in neutrophilic granulocytes, monocytes, and macrophages, comprises up to 60 % of the total cytosolic protein content of neutrophils, resists metabolic degradation and can be measured in feces (ref. 10-12). Its use as a biomarker of intestinal inflammation has been extensively validated, showing consistently abnormal levels in the stool of individuals with IBD (ref. 2, 13-16).

PRINCIPLE OF THE PROCEDURE

The BÜHLMANN fCAL® ELISA is a colorimetric, microtiter plate based enzyme linked immuno-sorbent assay that uses monoclonal antibodies (mAb) highly specific to calprotectin heterodimeric and polymeric complexes. Calprotectin present in the calibrators, controls and samples binds to the antibody coated on the wells. After a washing step, a monoclonal detection antibody conjugated to horseradish peroxidase (HRP) is added which binds specifically to the calprotectin molecules captured by the antibody bound to the well. Tetramethylbenzidine (TMB) is added as the enzyme substrate (blue color formation) followed by a stopping reaction (change to yellow color). The optical density of the developed color, measured at 450 nm, is proportional to the concentration of calprotectin in the calibrators, controls and samples.

REAGENTS SUPPLIED
COMPOSITION AND PREPARATION OF REAGENTS

Reagents	Quantity		Code	Reconstitution
	EK-CAL	EK-CAL2		
Extraction Buffer	3 bottles x 125 mL	6 bottles x 125 mL	B-CAL-EX	Ready to use
Microtiter Plate precoated with anti-calprotectin mAb	12 x 8 wells	2 x 12 x 8 wells	B-CAL-MP	Ready to use
Plate Sealer	3 pieces	6 pieces	-	Ready to use
Wash Buffer Concentrate (10x) with preservatives	1 bottle x 100 mL	2 bottles x 100 mL	B-CAL-WB	Dilute each with 900 mL of deionized H_2O
Incubation Buffer with preservatives	2 bottles x 125 mL	3 bottles x 125 mL	B-CAL-IB	Ready to use
Calibrators A to E [1] calprotectin in a buffer matrix with preservatives	5 vials x 1 mL	5 vials x 1 mL	B-CAL-CASET	Ready to use
Control Low / High [2] human serum with preservatives	2 vials x 1 mL	2 vials x 1 mL	B-CAL-CONSET	Ready to use
Enzyme Label anti-calprotectin mAb conjugated to HRP	1 vial x 12 mL	2 vials x 12 mL	B-CAL-EL	Ready to use
TMB Substrate TMB in citrate buffer	1 vial x 12 mL	2 vials x 12 mL	B-TMB12	Ready to use
Stop Solution 0.25 M sulfuric acid	1 vial x 12 mL	2 vials x 12 mL	B-STS12	Ready to use Corrosive agent

Table 1: Reagents supplied.

[1] The calibrators are ready to use. The actual calprotectin concentration of calibrators A to E are 4, 12, 40, 120 and 240 ng/mL, respectively. Fecal samples are extracted at a 1:50 dilution. An additional 1:150 dilution of the extracted sample (for a total dilution of 1:7500) is used for the measurement. To take these dilution steps into account for the final calculations, the following concentrations for calibrators A to E have to be used in the ELISA protocol:

30, 90, 300, 900 and 1800 µg calprotectin per 1 g of stool.

[2] Controls are ready to use. The controls contain lot specific amounts of native human calprotectin. Refer to the additional QC data sheet for actual concentrations.

MATERIALS REQUIRED BUT NOT PROVIDED

Extraction procedure

- 15 mL polypropylene tubes with screw caps (e.g. Sarstedt: #62.554.502)
- 10 µL single use inoculation loops, blue (e.g. Sarstedt: #86.1562.050)
- Precision balance 10-200 mg
- 100 and 1000 µL precision pipettes with disposable tips
- Vortex mixer
- Micro centrifuge
- 2 mL micro tube (e.g. Sarstedt: #72.691)
- Orbital shaker for microtiter plates, e.g MTS 2/4 digital, IKA®-Werke GmbH & Co. KG

ELISA procedure

- 10, 100 and 1000 µL precision pipettes with disposable tips
- Disposable polystyrene or polypropylene tubes for the preparation of sample dilutions
- 1000 mL cylinder for dilution of the wash buffer concentrate
- Microtiter plate washer (see procedural notes) or squeeze bottle for wash buffer
- Orbital shaker for microtiter plates
- Blotting paper
- Microtiter plate reader for measurement of absorbance at 450 nm

WARNINGS AND PRECAUTIONS

- This kit is for *in vitro* diagnostic use only.
- For information on hazardous substances included in the kit please refer to Material Safety Data Sheets available upon request directly from BUHLMANN Diagnostics Corp, BDC at (844) 300-9799 (Mon-Fri 8:00AM-5:00PM EST).
- The microtiter strips, calibrators and controls of this kit contain components of human origin. Each serum donor unit used in the preparation of the kit components was tested and found negative for HBV surface antigen, as well as for HCV and HIV1/2 antibodies. Although these methods are highly accurate, there is no guarantee that this material cannot transmit Hepatitis or AIDS. Therefore, all specimens and kit components should be handled as if capable of transmitting infections. All products containing human source material should be handled in accordance with good laboratory practice (GLP) using appropriate precautions.
- Read the instructions carefully prior to carrying out the test. Test performance will be adversely affected, if reagents are incorrectly diluted, modified or stored under conditions other than those as detailed in this instruction for use. Optimal test results are only obtained when using calibrated pipettes and microtiter plate readers.
- Do not mix or use components from kits with different lot numbers. It is advised not to exchange wells of different plates even of the same lot. The kits may have been shipped or stored under different conditions and the binding characteristics of the plates may produce slightly different results.
- Do not use reagents beyond expiry date as shown on the kit labels.
- Microwells cannot be re-used.
- Prepared or used reagents and chemicals have to be treated as hazardous waste according to the national biohazard safety guideline or regulation.
- The stop solution contains sulfuric acid. The reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothing. Wear suitable protective clothing, gloves and eye protection. After contact with eyes or skin, wash immediately with plenty of water.

  R43: May cause sensitization by skin contact. S28-37: After contact with skin, wash immediately with plenty of soap and water. Wear suitable gloves.
- Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes
- Do not smoke, eat, drink or apply cosmetics in areas where specimens or kit reagents are handled.
- Wear disposable latex gloves when handling specimens and reagents. Microbial contamination of reagents or specimens may give false results.
- Handling should be in accordance with the procedures defined by an appropriate national biohazard safety guideline or regulation.

Technical precautions

- Residues in the microtiter plate wells result from the production process. They are removed in the washing step (step 3 of the assay procedure) and do not affect the results.
- Let the reagents equilibrate to room temperature. Mix (vortex) the reagents well before use.

Extraction procedure

- To achieve quantitative results, it is important to completely homogenize the added stool sample in the extraction buffer. There may be a small amount of insoluble (undigested) particles remaining after mixing. The particles should not affect the results.
- Turbidity of the extract may still be observed after the 5 minute centrifugation step. The turbidity can be reduced by further centrifugation, but such turbidity has not been found to affect the quantitative results.

ELISA procedure

- In the ELISA procedure the washing steps are essential to guarantee reproducible results. Allow the wash buffer to incubate in the wells for a minimum of 20 seconds before removing.
- If an automated washer is used, "plate mode" is strongly recommended, i.e. each process step (dispense / aspiration) is carried out on all of the strips sequentially, before the instrument continues with the next washing cycle. Thus, the minimal incubation time is guaranteed.
- For information on washer settings contact BUHLMANN Diagnostics Corp, BDC at (844) 300-9799 (Mon-Fri 8:00AM-5:00PM EST).

- The indicated number of washing cycles (see below) is mandatory to ensure reproducible results.
- To ensure the antigen / antibody reaction is complete, the incubation time in step 5 must be at least 30 minutes. Moderately longer incubation time (up to 5 minutes) has no influence on the final outcome.
- Plate rotator (shaker) must be adjusted to approximately 450 rpm (<7.5 Hz). Higher rotation frequency may cause poor dilution linearity at values between 900 and 1800 µg/g. Orbital rotation instead of reciprocal shaking should be used.
- The enzyme label is inactivated by oxygen and is highly sensitive to sodium azide, thimerosal, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Therefore only deionized high quality water must be used.
- A standard curve with controls must be run with each new assay (each plate or partial plate).

STORAGE AND STABILITY OF REAGENTS AND WORKING SOLUTIONS

Unopened reagents	
Store at 2-8 °C. Do not use kit past expiration date printed on the labels.	
Opened / Reconstituted reagents	
Extraction Buffer	Store for up to 6 months at 2-8 °C.
Microtiter Plate	Return unused strips immediately to the foil pouch containing the desiccant packs and reseal along the entire edge of zip-seal. Store for up to 6 months at 2-8 °C.
Diluted Wash Buffer	Store for up to 6 months at 2-8 °C.
Incubation Buffer	
Calibrators	
Controls	
Enzyme Label	
TMB Substrate	
Stop Solution	

Table 2: Storage and stability of reagents

SAMPLE COLLECTION AND STORAGE

Collection of less than 1 g (1 mL) of stool sample is required. The extraction procedure requires 50-100 mg of stool sample.
Collect stool samples into plain tubes and store them refrigerated at 2-8 °C until ready for transport to the laboratory.
Important: The sample must be collected in empty collection devices; no chemical or biological additives should be added to the sample.

Specimen transport

Stool specimens should be received for processing by the laboratory within 3 days of collection. Stool specimens may be shipped at room temperature or on cold packs.

Specimen storage

Stool specimens should be refrigerated at 2-8 °C and extracted within 3 days of receipt at the laboratory. Do not store samples at elevated temperatures.

Extract storage

Fecal calprotectin extracts are stable refrigerated for up to 7 days at 2-8 °C. For longer storage, freeze the extracts at -20 °C. Frozen extracts are stable for a period of up to 36 months (3 years).

PROCEDURE

Specimen preparation –extraction procedure

Step 1: Label and weigh the empty polypropylene tube, including the inoculation loop. Note the weight (tare).

Step 2: Take out 50 to 100 mg of the stool sample by means of the inoculation loop and place it into the pre-weighted tube and weigh it again (gross weight). Avoid taking up dietary fibers present in the sample during the sampling process.

Step 3: Calculate the net amount of sample, by subtracting tare from the gross weight, break the inoculation loop away, and leave the lower part of the loop in the tube. Add extraction buffer (49 times the weight [in µls] of the sample) into the tube and cap the tube.

Step 4: Extract the samples by
- Vigorously vortex the extraction tube containing buffer and stool sample on a vortex mixer (at highest speed) for 30 secs.
- Incubate the extraction tube for 25 +/- 5 minutes on an orbital plate shaker at ca. 400 rpm. The inoculation loop inside the tube serves to enhance agitation.
- Again, vigorously vortex the extraction tube for 30 seconds.

Step 5: Transfer 1.5 mL of the homogenate into a 2 mL micro tube.

Step 6: Centrifuge the extract in the tube for 5 minutes at 3000 x g.

Step 7: Decant the supernatant into a fresh labeled tube and continue with the ELISA procedure, or store the extracts at 2-8 °C for ≤7 days or at ≤-20 °C for ≤36 months.

ELISA procedure

- Allow all reagents to equilibrate to 18-28 °C prior to use.
- Only dilute stool extracts. Calibrators and controls are ready to use.
- Let the samples equilibrate for at least 5 minutes at 18-28 °C prior to proceeding to step 4c.

Continuing from Step 7 of the extraction procedure, the assay can be performed according to the following ELISA procedure:

Step 1: Dilute the stool extract 1:150 with incubation buffer (B-CAL-IB) (e.g. 20 µL extract and 2980 µL incubation buffer) and mix well.

Step 2: Prepare a plate with sufficient strips to test the required number of calibrators, controls and diluted samples. Remove excess strips from the holder and re-seal them in the foil pouch together with the desiccant packs without delay. Store refrigerated.

Step 3: Wash the coated wells twice using at least 300 µL of wash buffer (B-CAL-WB) per well. Empty the wells and tap the plate firmly onto blotting paper.

Important: Allow wash buffer to remain in the wells for a minimum of 20 seconds during each wash step.
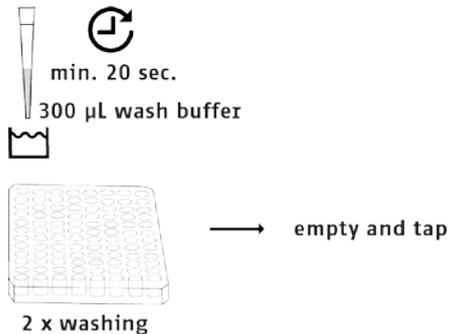
Step 4a: Pipet 100 µL of incubation buffer in duplicate into wells A1 + A2 (blank).

Pipet 100 µL of calibrators A-E (B-CAL-CASET) in duplicate into wells B1 + B2 through F1 + F2.

Step 4b: Pipet 100 µL of controls low and high (B-CAL-CONSET) in duplicate into wells G1 + G2 and H1 + H2, respectively.

Step 4c: Pipet 100 µL of each diluted sample into the subsequent wells (A3, B3, C3, D3…).

Step 5: Cover the plate with a plate sealer, and incubate for 30 to 35 minutes on an orbital plate shaker set to ~450 rpm at 18-28 °C (see technical precautions – ELISA procedure).

Step 6: Remove and discard the plate sealer. Empty the wells and wash three times using at least 300 µL of wash buffer per well (see technical precautions – ELISA procedure). Empty the wells and tap the plate firmly onto blotting paper.
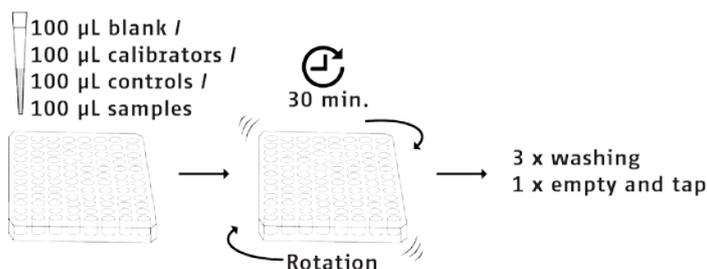
Step 7: Pipet 100 µL of enzyme label (B-CAL-EL) to all wells.

Step 8: Cover the plate with a plate sealer, and incubate for 30 ±5 minutes on an orbital plate shaker set to ~450 rpm at 18-28 °C.

Step 9: Remove and discard the plate sealer. Empty the wells and wash five times using at least 300 µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper.
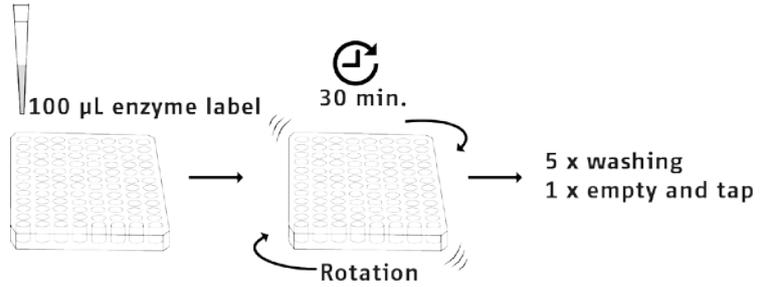
Step 10: Allow the TMB substrate solution to equilibrate to 18-28 °C.

Pipet 100 µL of TMB substrate solution (B-TMB12) to each well.

Step 11: Cover the plate with a plate sealer, protect the plate from direct light and incubate for 15 ± 2 minutes on a plate rotator set at ~450 rpm at 18-28 °C.
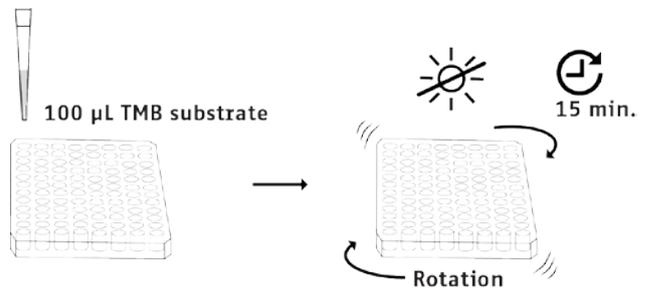
Step 12: Pipet 100 µL of stop solution (B-STS12) to all wells. Remove air bubbles with a pipette tip. Proceed to step 13 within 30 minutes.

Step 13: Read the absorbance at 450 nm in a microtiter plate reader.
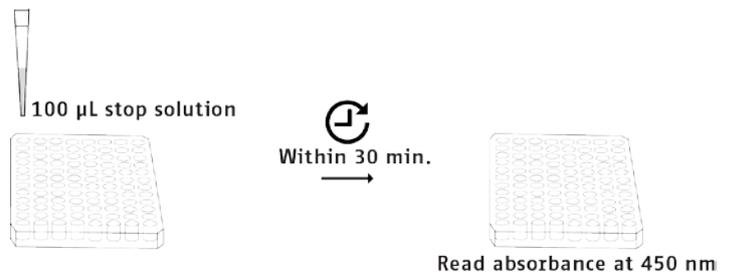
ASSAY CALIBRATION AND QUALITY CONTROL

Thorough understanding of this instruction for use is necessary for the successful use of the product. Reliable results will be obtained only by precise laboratory techniques (current GLP guidelines) and accurately following this instruction for use.

The BÜHLMANN fCAL® ELISA kit comes with two controls: controls low and high. The corresponding reference values of the controls are stated in the QC data sheet provided with each kit. The values and ranges stated on the QC data sheet always refer to the current kit lot and should be used for direct comparison of the results. Should the results for the controls low and/or high be out of the range stated in the QC data sheet, it is recommended to consider the whole run as invalid.

It is recommended to use internal control samples, in addition to kit controls, according to state and federal regulations. The use of internal control samples is advised to assure the day to day validity of results. Since there is no control serum for fecal calprotectin commercially available, we recommend using a pool of stool extracts with normal and pathological levels for internal quality control.

The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. If the precision of the assay does not meet the established limits and repetition has excluded errors in technique, check the following issues: i) pipetting, temperature controlling and timing devices; ii) ELISA reader settings; iii) expiration dates of reagents; iv) storage and incubation conditions; v) TMB substrate solution should be colorless; vi) purity of water; vi) aspiration and washing methods.

Participation in national or international external quality assessment programs is recommended as additional quality control.

Employ appropriate statistical methods for analyzing control values and trends. If the results of the assay do not meet the established acceptable ranges of control materials, results should be considered invalid. After checking the above mentioned items without finding any error, contact BUHLMANN Diagnostics Corp, BDC directly at (844) 300-9799 (Mon-Fri 8:00AM-5:00PM EST).

Reportable range

30 - 1800 µg/g (4 - 240 ng/mL)

CALCULATION OF TEST RESULTS

Read the absorbance at 450 nm in a microplate reader for the calibrator duplicates, control duplicates and samples.

Standard curve

It is recommended to use a software program capable of the following calculations; subtract the average blank OD value from each calibrator well. Calculate the average OD value for each calibrator. Establish a standard curve using a 4 parameter logistic (4 PL) fit.
The following calibrator concentrations have to be used in the ELISA protocol: 30, 90, 300, 900 and 1800 µg/g calprotectin.

Controls

It is recommended to use a software program capable of the following calculations; subtract the average blank OD value from each control well. Calculate the calprotectin concentration of the control in each well, in µg/g, using the established standard curve. Average the duplicate concentration values to get the calprotectin concentration of the controls in µg/g.

Samples

It is recommended to use a software program capable of the following calculations; subtract the average blank OD value from each sample well. Calculate the calprotectin concentration of each sample in µg/g, using the established standard curve.

Note: Refer to Figure 1 and Table 3 for typical data results and standard curve. These results and standard curve are provided for demonstration purposes only. A standard curve must be generated for each set of samples to be assayed.
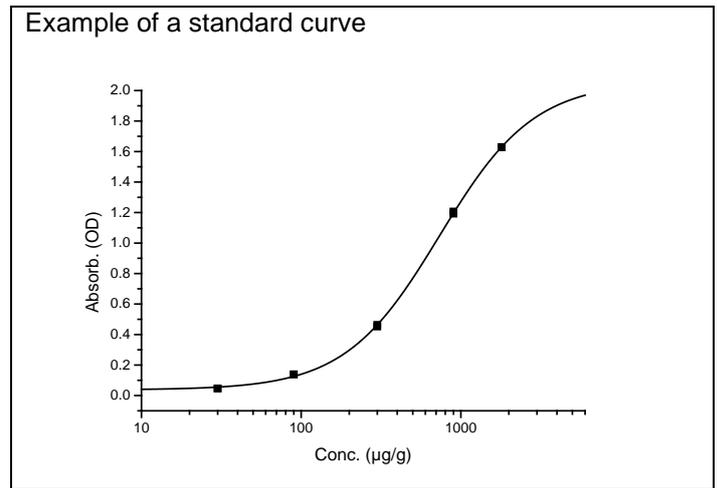
Figure 1: Example of a standard curve

	Concentration [µg/g]	Absorbtion [OD]
Calibrator A	30 30	0.047 0.046
Calibrator B	90 90	0.138 0.140
Calibrator C	300 300	0.464 0.452
Calibrator D	900 900	1.207 1.192
Calibrator E	1800 1800	1.627 1.630
Blank avg.		0.057
Ctrl low		0.147 0.162
Ctrl high		0.618 0.618

Table 3: Example results

LIMITATIONS

- Test results should be interpreted in conjunction with information available from clinical assessment of the patient and other diagnostic procedures.

- False negative results could occur in patients who have granulocytopenia due to bone marrow depression.

- Some patients taking non-steroidal anti-inflammatory drugs (NSAID) will have elevations in their fecal calprotectin levels.

- Results may not be clinically applicable to children less than 4 years of age who have mildly increased fecal calprotectin levels.

- Patients with IBD fluctuate between active (inflammatory) and inactive stages of the disease. These stages must be considered when interpreting results of the fecal calprotectin assay.

- Other intestinal diseases, including many gastrointestinal infections and colorectal cancer, can result in elevated levels of calprotectin. Therefore, a diagnosis of active IBD should be made only in the context of other diagnostic testing and the total clinical status of the patient.
- Fecal calprotectin is an indicator of the presence of neutrophils in the stool and is not specific for IBD.

CLINICAL THRESHOLDS

Results from 58 clinical samples from patients diagnosed with IBS and 131 clinical samples from patients diagnosed with IBD, from an international clinical study, were analyzed to obtain the values described in Table 4.

Calprotectin concentration	Interpretation	Follow-up
< 80 µg/g	Normal	None
80 - 160 µg/g	Gray-zone/Borderline	Follow-up within 4 – 6 weeks
> 160 µg/g	Elevated	Repeat as needed

Table 5: BÜHLMANN fCAL® ELISA diagnostic ranges.

Calprotectin values below 80 µg/g

Fecal calprotectin values <80 µg/g are not indicative of active inflammation in the gastrointestinal tract. Low fecal calprotectin levels (calprotectin <80 µg/g) can be used in conjunction with the patient's clinical symptoms, medical history, and other clinical and laboratory findings to determine the need for additional diagnostic work-up. Specifically, for patients with a clinical and laboratory presentation suggesting a non-inflammatory disorder such as IBS fecal calprotectin values of <80 µg/g can be used to support a decision to defer invasive testing.

Calprotectin values between and equal to 80 and 160 µg/g

Mid-fecal calprotectin levels between and equal to 80 and 160 µg/g, also called gray-zone levels, are not directly indicative of an active inflammation requiring immediate follow-up with invasive testing. However, the presence of inflammation cannot be excluded. Re-evaluation of fecal calprotectin levels after 4 to 6 weeks is recommended to determine the inflammatory status. This decision should be made by the clinician in conjunction with the patient's clinical symptoms, medical history, and other clinical and laboratory findings.

Calprotectin values greater than 160 µg/g

Fecal calprotectin values >160 µg/g are indicative of neutrophil infiltrate in the gastrointestinal tract; therefore, this may signal the presence of active inflammatory disease. Elevated fecal calprotectin levels (calprotectin >160 µg/g) can be used in conjunction with the patient's clinical symptoms, medical history, and other clinical and laboratory findings to determine the need for further investigative procedures, including invasive procedures performed by specialists, to achieve an overall clinical diagnosis, in particular of IBD.

CLINICAL EVALUATION

The ability of the BÜHLMANN fCAL® ELISA to discriminate between patients with IBD and other non-inflammatory GI disorders, including IBS, was tested in a clinical study with a total of 337 adult and pediatric patients. One hundred and thirty five patients (135) had a final diagnosis of IBD (Crohn's disease, ulcerative colitis or indeterminate colitis), 130 patients suffered from IBS and 72 patients presented with abdominal pain and/or diarrhea, or other GI-related non-inflammatory conditions. Final diagnosis was supported by endoscopic as well as other clinical findings.

Final diagnosis	Distribution of patients' results in numbers (percent) withn BÜHLMANN fCAL ELISA diagnostic ranges.			
	< 80 µg/g	80 - 160 µg/g	> 160 µg/g	Total
IBD	9 (6.7%)	12 (8.9%)	114 (84.4%)	135 (100%)
IBS	94 (72.3%)	17 (13.1%)	19 (14.6%)	130 (100%)
Other GI	48 (66.7%)	10 (13.9%)	14 (19.4%)	72 (100%)

Table 6: Distribution of patients' results within BÜHLMANN fCAL ELISA diagnostic ranges.

IBD vs. non-IBD	Clinical decision point	
	80 µg/g	160 µg/g
Sensitivity (95% CI)	93.3% (87.7%, 96.9%)	84.4% (77.2%, 90.1%)
Specificity (95% CI)	70.3% (63.5%, 76.5%)	83.7% (77.8%, 88.5%)
PPV (95% CI)	67.7% (60.5%, 74.4%)	77.6% (69.9%, 84.0%)
NPV (95% CI)	94.0% (89.0%, 97.2%)	88.9% (83.6%, 93.0%)
ROC AUC (95% CI)	0.923 (0.893, 0.953)	

Table 7: Clinical performance characteristics of the BÜHLMANN fCAL® ELISA in discriminating IBD from non-IBD – IBS and other GI-related disorders, at 80 µg/g and 160 µg/g clinical decision points

IBD vs. IBS	Clinical decision point	
	80 µg/g	160 µg/g
Sensitivity (95% CI)	93.3% (87.7%, 96.9%)	84.4% (77.2%, 90.1%)
Specificity (95% CI)	72.3% (63.8%, 79.8%)	85.4% (78.1%, 91.0%)
PPV (95% CI)	77.8% (70.6%, 83.9%)	85.7% (78.6%, 91.2%)
NPV (95% CI)	91.3% (84.1%, 95.9%)	84.1% (76.7%, 89.9%)
ROC AUC (95% CI)	0.933 (0.902, 0.963)	

Table 8: Clinical performance characteristics of the BÜHLMANN fCAL® ELISA in discriminating IBD from IBS at 80 µg/g and 160 µg/g clinical decision points

CI – confidence interval

PPV – positive predictive value

NPV – negative predictive value

ROC AUC – area under receiver operating characteristic curve

PERFORMANCE CHARACTERISTICS

Reproducibility (Multisite Precision Evaluation Study)

Reproducibility was established according to the CLSI guideline EP05-A3 by performing measurements at three routine laboratory sites. Five pooled stool specimen extracts with calprotectin concentrations covering the measuring range of the test and clinical decision points were tested over five days, in two runs per day, with four results generated per run. The series of measurements was performed independently by two operators at each study site, alternating in each run between two different ELISA readers and two different reagent lots. Three reagent lots, in total, were used across the study sites.

ID	Mean [µg/g]	n	Within-run SD	Within-run %CV	Between-run SD	Between-run %CV	Between-day SD	Between-day %CV
S01	41.4	240	3.1	7.4%	8.1	19.6%	0.0	0.0%
S02	67.2	240	3.0	4.4%	7.0	10.4%	0.0	0.0%
S03	143.0	240	5.6	3.9%	15.7	11.0%	0.0	0.0%
S04	379.8	240	10.8	2.9%	16.4	4.3%	3.2	0.9%
S05	1056.6	240	40.1	3.8%	53.8	5.1%	0.0	0.0%

Table 9: Reproducibility study results – within-run, between-run and between-day variance component estimates

ID	Mean [µg/g]	n	Between-operator SD	Between-operator %CV	Between-site SD	Between-site %CV	Total (Reproducibility) SD	Total (Reproducibility) %CV
S01	41.4	240	0.0	0.0%	4.1	9.9%	9.6	23.2%
S02	67.2	240	1.9	2.8%	3.9	5.8%	8.8	13.0%
S03	143.0	240	2.6	1.8%	3.7	2.6%	17.3	12.1%
S04	379.8	240	0.0	0.0%	13.7	3.6%	24.2	6.4%
S05	1056.6	240	39.6	3.7%	67.6	6.4%	103.1	9.8%

Table 10: Reproducibility study results – between operator, between-site and total precision estimates

Within-laboratory precision

Repeatability and within-laboratory precision were established according to the CLSI guideline EP05-A3. Seven pooled stool specimen extracts with calprotectin concentrations covering the measuring range of the test and clinical decision points were tested over ten days, in two runs per day, with four results generated per run. One reagent lot was used in the study.

ID	Mean [µg/g]	n	Repeatability SD	Repeatability %CV	Between-run SD	Between-run %CV	Between-day SD	Between-day %CV	Within-laboratory SD	Within-laboratory %CV
P1	38.5	80	2.3	5.8%	1.8	4.8%	2.2	5.6%	3.6	9.4%
P2	67.0	80	2.0	3.0%	3.5	5.2%	1.6	2.4%	4.3	6.4%
P3	135.7	80	2.3	1.7%	5.6	4.1%	0	0%	6.0	4.4%
P4	207.1	80	4.1	2.0%	12.5	6.0%	0	0%	13.2	6.4%
P5	337.1	80	5.9	1.8%	18.3	5.4%	0	0%	19.3	5.7%
P6	562.6	80	11.0	2.0%	13.6	2.4%	2.5	0.4%	17.7	3.1%
P7	918.0	80	18.6	2.0%	62.1	6.8%	20.8	2.3%	68.1	7.4%

Table 11: Within-laboratory precision study results

Extraction reproducibility

Nine clinical stool specimens, selected to reflect different stool consistencies: solid, semi-solid and liquid, with calprotectin concentrations covering the measuring range of the test and clinical decision points, were extracted ten times each. Each stool extract was tested in duplicate using one reagent lot of the BÜHLMANN fCAL® ELISA.

ID	Mean [µg/g]	n	Within-extraction SD	Within-extraction %CV	Between-extraction SD	Between-extraction %CV	Total precision SD	Total precision %CV
S01	51.2	20	4.5	8.9%	7.8	15.2%	9.0	17.6%
S03	88.3	20	6.6	7.5%	13.3	15.0%	14.8	16.8%
S05	66.8	20	10.6	15.8%	3.7	5.5%	11.2	16.7%
S06	179.3	20	16.8	9.4%	32.8	18.3%	36.8	20.5%
S07	366.1	20	22.4	6.1%	32.3	8.8%	39.3	10.7%
S08	327.4	20	15.4	4.7%	26.9	8.2%	31.0	9.5%
S09	1783.7	20	198.3	11.1%	262.0	14.7%	328.6	18.4%

Table 12: Extraction reproducibility study results

Accuracy / Recovery

Seven stool specimen extracts with calprotectin levels spanning the measuring range of the test were spiked with 180 µg/g calprotectin in calibrator material. Spiking was performed at 10 % the specimen extract volume. "Baseline" samples were spiked with the corresponding amount of incubation buffer. "Baseline" and "baseline + spike" samples were measured in three replicates with one reagent lot.

ID	Mean baseline [µg/g]	Expected baseline + spike [µg/g]	Observed baseline + spike [µg/g]	Recovery rate [%]
#1	46.5	226.5	224.5	99.1%
#2	63.7	243.7	247.7	101.6%
#3	89.0	269.0	274.9	102.2%
#4	111.6	291.6	292.0	100.1%
#5	163.5	343.5	331.1	96.4%
#6	304.0	484.0	475.0	98.1%
#7	990.2	1170.2	1166.6	99.7%

Table 13: Accuracy/recovery study results

Limit of Blank (LoB): 8.3 µg/g

The LoB was established according to the CLSI guideline EP17-A2 with four negative (incubation buffer spiked with extraction buffer) samples. The samples were measured over three days in five replicates each day to produce 60 blank values. The LoB was calculated using non-parametric analysis. The study was performed independently with two reagent lots, taking the higher estimate obtained with one lot as the claimed LoB value.

Limit of Detection (LoD): 12.6 µg/g

The LoD was established according to the CLSI guideline EP17-A2 with four stool specimen extracts with concentrations corresponding to 1 - 5 times the LoB value. The samples were measured over three days in five replicates each day to produce 60 values. The LoD was calculated using parametric analysis. The study was performed independently with two reagent lots, taking the higher estimate obtained with one lot as the claimed LoD value.

Limit of Quantitation (LoQ): 30 µg/g

The LoQ was established according to the CLSI guideline EP17-A2 with four low level stool specimen extracts. The samples were measured over three days in five replicates each day to produce 60 values. The LoQ was defined as the lowest calprotectin concentration, which can be determined with a precision of below 20 % CV.

Linearity

The linear range of the BÜHLMANN fCAL® ELISA was determined according to the CLSI guideline EP06-A. To demonstrate matrix linearity, two dilution series, with at least 14 different calprotectin levels, covering the expected measuring range of the test, were generated by diluting high stool specimen extracts with incubation buffer. To demonstrate aqeous linearity, calibrator E was titred with incubation buffer to generate nine samples covering the expected measuring range of the test. Each sample was tested in three replicates. The linear range was defined as the concentration interval in which coefficients of the non-linear, polynomial, fits were determined as not significant or as the concentration interval in which the deviation of the polynomial fit from linearity was below 20 %. For values below 75 µg/g an absolute difference of less than 15 µg/g was allowed.

ID	Matrix/ aqeous	Measuring range tested	R^2	p-value for non-linear coefficient	Linear range
FRB	matrix	22.8 – 1932.0	0.998	$p < 0.05$	24.6 – 1932.0
FRC	matrix	26.2 – 2096.2	0.997	$p < 0.05$	26.2 – 2096.2
FRD	aqeous	39.0 – 1830.2	0.994	$p > 0.05$	39.0 – 1830.2

Table 14: Linearity study results

Interfering substances

The susceptibility of the BÜHLMANN fCAL® ELISA assay to oral pharmaceuticals, nutritional supplements, hemoglobin as well as entheropathological microorganisms was assessed according to the CLSI guideline EP07-A2. Bias in results exceeding 10 % was considered interference. No interference was detected with substances, listed in Table 15, up to the indicated concentrations. No interference was detected with entheropathological microorganisms, listed in Table 16, up to the indicated amounts of colony forming units (CFU) per mL of stool specimen extract.

Trade Name	Active Component	Concentration mg/50 mg stool
gyno-Tardyferon	Iron (II) sulfate (contains 0.35 mg folic acid)	0.11
Prednisone	Prednisone	0.31
Imurek	Azathioprine	0.19
Salofalk	Mesalamine; 5-ASA	5.21
Agopton	Lansoprazole	0.18
Asacol	Mesalamine; 5-ASA	2.50
Vancocin	Vancomycin	2.00
Sulfamethoxazole	Sulfamethoxazole	1.60
Trimethoprim	Trimethoprim lactate	0.35
Ciproxine	Ciprofloxacin	1.25
Vitamin E	DL-α-Tocopherol Acetate	0.30
Bion 3	3 probiotics (107 CFU): lactobacillus gasseri PA16 / 8, bifidobacterium bifidum MF 20/5, bifidobacterium longum SP07 / 3, 12 vitamins: A (800 µg), B1 (1.4 mg), B2 (1.6 mg), B6 (2 mg), B12 (1 µg), C (60 mg), D (5 µg), E (10 mg), Biotin (150 µg), folic acid (200 µg), niacin (18 mg), pantothenic acid (6 mg) and 7 minerals: iodine (100 µg), iron (5 mg), zinc (5 mg), selenium (30 µg), chromium (25 µg), manganese (1.2 mg), molybdenum (25 µg)	1.06
Hemoglobin	Hemoglobin	1.25

Table 15: Interfering substances: oral pharmaceuticals, nutritional supplements, hemoglobin

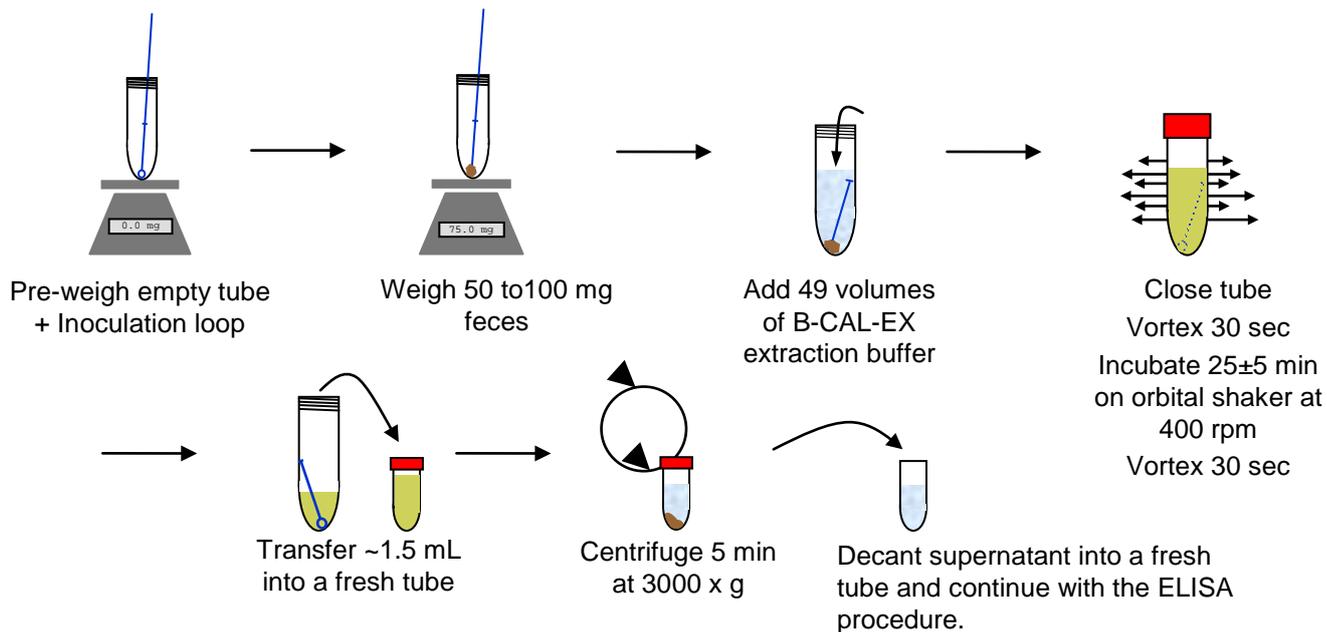| Name	Final Concentration (CFU/mL)
Escherichia coli	9.5×10^7
Salmonella enterica subsp. enterica	1×10^9
Klebsiella pneumoniae subsp. pneumonia	5.4×10^7
Citrobacter freundii	9.7×10^7
Shigella flexneri	1.5×10^8
Yersinia enterocolitica subsp. enterocolitica	1.6×10^8

Table 16: Interfering substances: entheropathological microorganisms
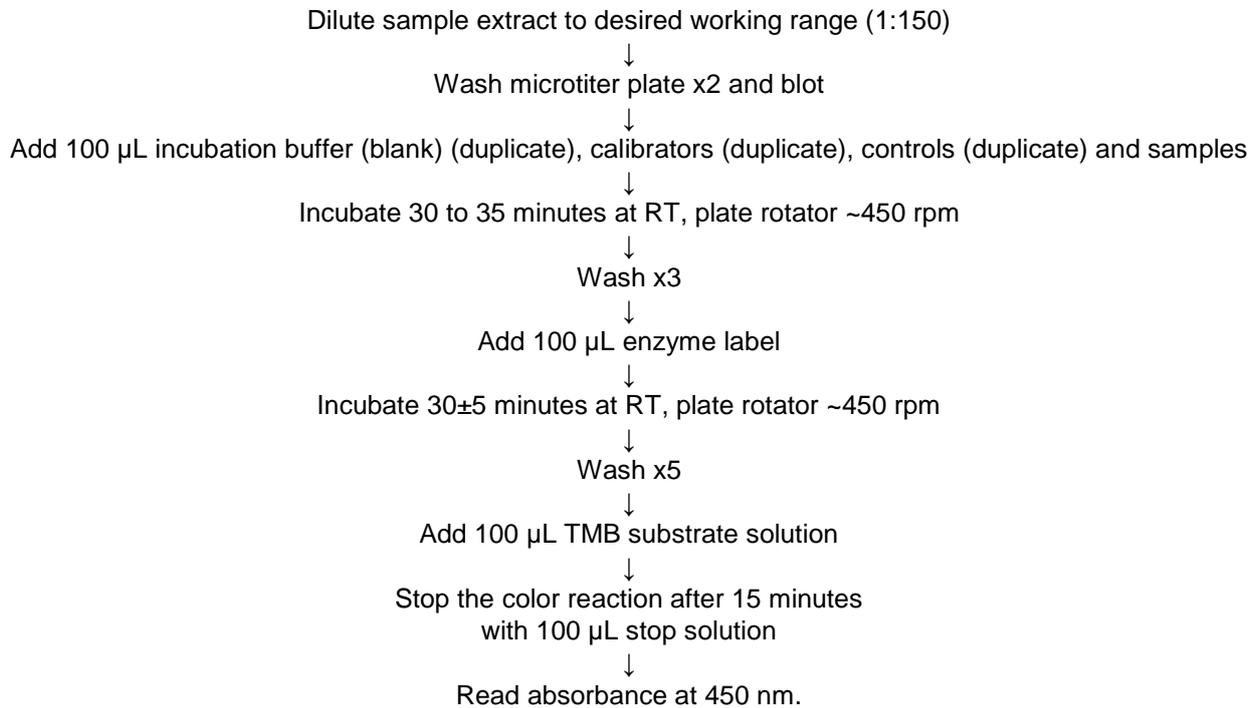
APPENDIX I

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APPENDIX II

SHORT TEST PROTOCOL

EXTRACTION PROCEDURE

Pre-weigh empty tube
+ Inoculation loop

Weigh 50 to 100 mg
feces

Add 49 volumes
of B-CAL-EX
extraction buffer

Close tube
Vortex 30 sec
Incubate 25±5 min
on orbital shaker at
400 rpm
Vortex 30 sec

Transfer ~1.5 mL
into a fresh tube

Centrifuge 5 min
at 3000 x g

Decant supernatant into a fresh
tube and continue with the ELISA
procedure.

↓

ELISA PROCEDURE

Dilute sample extract to desired working range (1:150)
↓
Wash microtiter plate x2 and blot
↓
Add 100 µL incubation buffer (blank) (duplicate), calibrators (duplicate), controls (duplicate) and samples
↓
Incubate 30 to 35 minutes at RT, plate rotator ~450 rpm
↓
Wash x3
↓
Add 100 µL enzyme label
↓
Incubate 30±5 minutes at RT, plate rotator ~450 rpm
↓
Wash x5
↓
Add 100 µL TMB substrate solution
↓
Stop the color reaction after 15 minutes
with 100 µL stop solution
↓
Read absorbance at 450 nm.

APPENDIX III

SYMBOLS USED

Symbol	Explanation	Symbol	Explanation
⧗	Use by	BUF WASH 10X	Wash Buffer Concentrate (10x)
REF	Catalogue number	BUF INC	Incubation Buffer
LOT	Batch code	CAL A – CAL E	Calibrator A-E
IVD	*In Vitro* Diagnostic Medical Device	CONTROL L	Control low
∑▽	Content sufficient for <n> tests	CONTROL H	Control high
📖	Consult instructions for use	EL	Enzyme Label
∮	Temperature limitation	SUBS TMB	TMB Substrate
MP	Microtiter plate	SOLN STOP	Stop Solution

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Manufactured by:
BÜHLMANN Laboratories AG
Baselstrasse 55
4124 Schönenbuch
Switzerland

Technical Service (U.S. & Canada Only):
(844)300-9799

Technical Service (Outside the U.S.):
+41 61 487 1212
info@buhlmannlabs.ch

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