Calprotectin Chemiluminescence ELISA

For *In Vitro* Diagnostic Use in the United States and Canada

REF **80-CALPHU-CH01** Σ 96 **Rx Only**

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Intended use

The ALPCO Calprotectin Chemiluminescence ELISA is an *in vitro* diagnostic chemiluminescent assay intended for the quantitative measurement of fecal calprotectin, a neutrophilic protein that is a marker of intestinal mucosal inflammation, in human stool. The ALPCO Calprotectin Chemiluminescence ELISA is intended for *in vitro* diagnostic use as an aid in the diagnosis of inflammatory bowel disease (IBD), specifically Crohn's disease (CD) and ulcerative colitis (UC), and as an aid in the differentiation of IBD from irritable bowel syndrome (IBS) in conjunction with other clinical and laboratory findings. For *in vitro* diagnostic use in the United States and Canada.

Summary and Explanation of the Test

Calprotectin, a heterodimer comprised of S100A8/A9 monomers, also known as myeloid-related protein (MRP) 8/14 subunits, is an immunomodulatory protein accounting for up to 60% of cytosolic protein in neutrophil granulocytes and macrophages. Calprotectin plays a central role in neutrophil defenses and inflammatory pathologies. Under conditions of intestinal inflammation, calprotectin is released into the gastrointestinal lumen and serves as an accurate biomarker of mucosal inflammation.[1]

Measurement of fecal calprotectin levels provides valuable information that can assist physicians to determine whether to send inflammatory bowel disease (IBD) patients for colonoscopy or treat them for IBS symptoms.[2] Traditionally, IBD activity has been assessed through clinical symptoms, but it has been reported that these recognized activity indexes are subjective and do not correlate as consistently as fecal calprotectin levels with histologic inflammation.[3]

Principles of the Procedure

The ALPCO Calprotectin Chemiluminescence ELISA is an enzyme linked immuno-sorbent sandwich assay system with chemiluminescence detection based on the use of monoclonal antibodies against calprotectin. A mouse IgG monoclonal capture antibody (mAb) highly specific to the calprotectin heterodimeric and polymeric complexes is coated onto the microtiter plate. Calprotectin present in the diluted sample is bound by the antibody adsorbed to the surface of the plastic well. Calibrators, controls and specimen extracts are incubated. After a washing step, a biotinylated secondary mouse IgG monoclonal detection antibody detects the calprotectin molecules bound to the antibody coated onto the plate. After incubation and a further washing step, a Streptavidin-Horseradish Peroxidase Enzyme conjugate binds to the available biotin on the immobilized secondary antibody. A chemiluminescent substrate is added and read when the substrate glows as a result of its oxidation with the enzyme. The signal is then read on a chemiluminescence plate reader. The intensity of the light is proportional to the amount of conjugate bound, and thus to the amount of captured calprotectin. Concentration of calprotectin in the samples is calculated using the provided Calibrators.

Reagents Supplied for 96 wells

Component	Quantity
Calprotectin Microplate	12 x 8 strips
Standards A-H	1 vial each
Control Levels 1-3	1 vial each
Sample Buffer (10X)	25 mL
Detector Antibody (101X)	150 µL
Detector Antibody Buffer	15 mL
Wash Buffer Concentrate (21X)	100 mL
Chemiluminescence Substrate A	6 mL
Chemiluminescence Substrate B	6 mL
Plate Sealers	3

Composition of Supplied Reagents/Materials

1. **Antibody-coated plate**
   12 x 8 wells coated with mouse monoclonal antibody against calprotectin. Foil bag with desiccant.
2. **Standards**
   8 vials containing calprotectin at eight concentrations (0, 5, 20, 40, 156, 625, 2500, 10000 µg/g). Ready-to-use.
3. **Controls 1-3**
   3 vials containing calprotectin. Ready-to-use.. The range of values for each control level are printed on the lot-specific Certificate of Analysis.
4. **Sample Buffer (10X)**
   1 bottle containing 25 mL of concentrated sample buffer.
5. **Detector Antibody (101X)**
   1 vial containing concentrated mouse monoclonal antibody against calprotectin to be diluted in detector antibody buffer.
6. **Detector Antibody Buffer**
   1 bottle containing 15 mL detector antibody buffer. Ready-to-use.
7. **Wash Buffer Concentrate (21X)**
   1 bottle containing 100 mL of concentrated wash buffer.
8. **Chemiluminescence Substrate A**
   1 bottle containing 6 mL substrate A. Ready-to-use.
9. **Chemiluminescence Substrate B**
   1 bottle containing 6 mL substrate B. Ready-to-use.

10. **Plate sealers**
    3 plate sealers. Ready-to-use.

Materials Required but Not Provided

Feces sample collection
1. Sample collection tube
2. Transport container

Feces preparation
1. Disposable, breakable sterile inoculation loops or wooden stick
2. Disposable polypropylene screw cap tubes, 14 ml
3. Eppendorf tubes (1 - 1.5 ml)
4. Sensitive digital scale (40-150 mg)
5. Extraction buffer (10-EXBUF-225)
6. Vortex mixer
7. Shaker
8. Centrifuge (1000 – 3000 x *g*)
9. Freezer (-20ºC, -80ºC)
10. ALPCO Easy 2 Stool Extraction Device (80-EZEX2-100) or the IDK Extract® Stool Collection Tube (K6999.US in the U.S. and K6999.C.100 in Canada: *formerly Easy Stool Extraction Device 30-EZEX-100*)

Equipment for ELISA measurements
1. Precision pipettes for dispensing up to 100 µL (with disposable tips)
2. Repeating or multi-channel pipette for dispensing up to 100 µL
3. Volumetric containers and pipettes for reagent preparation
4. Distilled or deionized water for reagent preparation
5. Automated microplate washer or hand-held manifold. A standard multi-channel pipette should not be used for washing.
6. Microplate shaker capable of 700-900 rpm
7. Microplate reader capable of reading luminescence

Precautions and Warnings

1. For *in vitro* diagnostic use in the United States and Canada.
2. Follow universal precautions. Materials of human origin used in this kit have been tested and confirmed negative for HBsAg and anti-HIV I and II and anti-HCV antibodies. They should be treated as a potential biohazard and handled and disposed of according to local laboratory legislation.
3. Reagents, samples and microtiter strips should be allowed to reach room temperature (18-28ºC) before starting the test.

4. Warning: do not interchange components from the different kit lots. Satisfactory performance of the test is guaranteed only when components from the same batch of the ALPCO Calprotectin Chemiluminescence ELISA are used.
5. Unused microtiter strips should be re-sealed airtight in the foil bag with the enclosed desiccant and stored at 2-8ºC.
6. Insufficient washing of the ELISA plate can lead to erroneous values of Calprotectin due to incomplete removal of reagents. Routine maintenance of aspiration/wash system is strongly recommended.
7. All reagents, except the detector antibody, streptavidin-HRP, streptavidin-HRP buffer, and chemiluminescence substrates, contain ProClin 300 as a preservative agent (< 0.10%).
8. Stool extraction devices should not be used for the extraction of liquid/watery stool samples (type 7 on the Bristol scale). Samples of this consistency should be extracted using the manual weighing extraction method.

Storage Conditions

1. All reagents and working solutions must be stored at 2-8ºC.
2. The expiration date is printed on all component labels.
3. Avoid exposure to high temperature, direct sunlight or extreme humidity.
4. Unused microtiter strips should be resealed airtight in the foil bag with the desiccant inside and stored at 2-8ºC.

Reagent Stability

Unopened Reagents
Store at 2-8ºC. Unopened reagents are stable until the expiration date printed on the label.
Opened Reagents
Store at 2-8ºC. Opened reagents are stable for up to 7 days.

Specimen Collection

Loose or liquid stool samples are acceptable as normalization to stool weight is part of the calculation of the result. Submission of stool samples from diapers should be avoided unless the sample submitted can be taken from a portion of the stool which is not in contact with the diaper material.

Sample requirements:

1. 1-5 g stool in a screw-top clean vial. No preservative is necessary or indicated.

2. Sample transport: Stool specimen should be received by the laboratory within 4 days of collection. Temperature during shipment should not exceed 28ºC. Samples must be stored at 2-8ºC upon receipt and extracted or frozen within 14 days of collection.
3. Sample storage: Samples may be stored at 2-8ºC for up to 14 days before extraction and testing. If samples will not be tested within 14 days, freeze samples at -20ºC or -80ºC. Freezing at -80ºC is recommended for long-term storage. Stool samples may be subject to no more than 3 freeze/thaw cycles.

Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 28ºC) and mix well.
2. Sample Buffer is to be diluted with 9 parts distilled or deionized water. For example, to prepare Working Strength Sample Buffer, dilute 19 mL of Sample Buffer Concentrate (10X) with 171 mL of deionized water.
3. Wash Buffer is to be diluted with 20 parts distilled or deionized water. For example, to prepare Working Strength Wash Buffer, dilute 20 mL of Wash Buffer Concentrate (21X) with 400 mL of deionized water.
4. Detector Antibody is to be diluted with 100 parts Detector Antibody Buffer. For example, to prepare enough Working Strength Detector Antibody for one complete microplate, dilute 120 µL of Detector Antibody Stock (101X) with 12 mL of Detector Antibody Buffer.
5. Chemiluminescence Substrates A & B are provided individually and should be combined in equal parts to create the Working Strength Chemiluminescence Substrate prior to use. For example, to prepare enough substrate for one complete microplate, combine 6 mL Chemiluminescence Substrate A with 6 mL Chemiluminescence Substrate B.
6. Standards and Controls are provided in a ready to use form. Please refer to the Certificate of Analysis for the standard concentrations and control ranges.
7. A standard curve must be performed with each assay run and with each microplate if more than one is used at a time. All standards and controls should be run in duplicate.

Sample Preparation – Extraction Procedure

Stool samples can be extracted using either the Stool Extraction Device method (see Instructions for Use provided with catalog number 80-EZEX2-100, K6999.US, or K6999.C.100) or the Manual Weighing/Standard Extraction procedure described below:

Important: The ALPCO Easy Stool Extraction method should not be used for the extraction of liquid stool samples.

1. Label and weigh (tare) the empty polypropylene tube together with the inoculation loop.
2. Take out 50 to 100 mg of the stool sample by means of the inoculation loop and place it into the pre-weighed tube.
3. Determine the net amount of sample. Break off the inoculation loop and leave the lower part of the loop in the tube. Add Extraction Buffer using 99 times the weight volume (1 X working dilution of 10-EXBUF-225) to the tube and close the tube.
4. Homogenize the sample on a multi-tube vortex by vigorous shaking (at highest speed) for 30 minutes.
5. Centrifuge the extract in the tube for 5 minutes at 3000 x *g*.
6. Decant the supernatant into a fresh labeled tube and continue with the assay procedure or store the extracts at 2-8°C for up to 3 days or at -80°C for up to 14 days. Stool sample extracts may be subject to no more than 3 freeze/thaw cycles. This extraction procedure results in a 1:100 dilution.

Assay Procedure

1. Dilute the stool extract 1:250 with working strength sample buffer (e.g. 10 µL extract and 2490 µL Sample Buffer) and mix well. This results in a final dilution of 1:25,000.
2. Prepare a plate with enough 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 as soon as possible. Store refrigerated.
3. Example plate layout is shown below. Fit the strip holder with the required number of micro ELISA strips. Use uncoated strips to complete the strip holder if the washer requires a full plate. Calibrators and controls must be included in each run.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std A	Std A	Ctrl 1	Ctrl 1	sample	sample	sample	sample	sample	sample	sample	sample
B	Std B	Std B	Ctrl 2	Ctrl 2	sample	sample	sample	sample	sample	sample	sample	sample
C	Std C	Std C	Ctrl 3	Ctrl 3	sample	sample	sample	sample	sample	sample	sample	sample
D	Std D	Std D	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
E	Std E	Std E	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
F	Std F	Std F	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
G	Std G	Std G	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample
H	Std H	Std H	sample	sample	sample	sample	sample	sample	sample	sample	sample	sample

4. Pipet 100 µL of each standard in duplicate into wells (i.e. A1-A2, B1-B2, etc.).

5. Pipet 100 µL of each control in duplicate into wells (i.e. A3-A4, B3-B4, etc.).

6. Pipet 100 µL of each diluted sample into wells (i.e. D3, D4, etc.).

7. Cover the plate with a plate sealer, and incubate for 30 ± 5 minutes on a plate shaker set at 700 - 900 rpm at 18 - 28°C.

8. Remove and discard the plate sealer. Empty the wells and wash six times using at least 300 µL of working strength wash buffer (see *Reagent Preparation*) per well. Empty the wells and tap the plate firmly onto blotting paper.

9. Pipet 100 µL of the diluted detector antibody (see *Reagent Preparation*) into all wells.

10. Cover the plate with a plate sealer, and incubate for 30 ± 5 minutes on a plate shaker set at 700 - 900 rpm at 18 - 28°C.

11. Remove and discard the Plate Sealer. Empty the wells and wash six times using at least 300 µL of diluted Wash Buffer (see *Reagent Preparation*) per well. Empty the wells and tap the plate firmly onto blotting paper.

12. Pipet 100 µL of the prepared Chemiluminescence Substrate Solution (see *Reagent Preparation*) into all wells.

13. Incubate for 2 to 5 minutes on a benchtop.

14. Read the plate immediately after incubation on a chemiluminescence microtiter plate reader using a 1 second (1000 ms) integration time.

Assay Calibration and Quality Control

1. A new standard curve is used with each run.

2. Controls 1, 2 and 3 are to be included in each run. The range for the controls is included on the enclosed Certificate of Analysis.

3. Traceability: No international reference material or reference measurement procedures are available for calprotectin. The Standards and Controls used in the ALPCO Calprotectin Chemiluminescence ELISA are traceable to internal standards made from native calprotectin antigen diluted in a buffer containing stabilizers and preservatives.

Reportable Range

7.9 – 6000 µg/g

Calculation of Results

Construct a standard curve from the standards. It is recommended to use a software program to calculate the standard curve and to determine the concentration of the samples.

The ALPCO Calprotectin Chemiluminescence ELISA is a ligand binding assay, with responses exhibiting a sigmoidal relationship to the analyte concentration. Currently accepted reference models for such curves use a 4- or 5-parameter logistic (pl) fit and a $1/y^2$ weighting scheme, as these models optimize the accuracy and precision across a greater range.

Samples can be read directly off the curve.

Interpretation of Results

A cut-off study was performed internally and provided the value reported in the table below:

Calprotectin Concentration	Interpretation	Follow-Up
< 50 µg/g	Normal	None
50 - 100 µg/g	Equivocal	Retest in 4-6 weeks
> 100 µg/g	Elevated	Repeat as clinically indicated

Limitations of the Procedure

- False-negative results could occur in patients who have granulocytopenia due to bone marrow depression.
- Some patients who are taking non-steroidal anti-inflammatory drugs (NSAID) will have elevations in their fecal calprotectin levels.[4]
- Patients with IBD fluctuate between active (inflammatory) and inactive stages of the disease. These stages must be considered when using the ALPCO Calprotectin Chemiluminescence ELISA.
- The use of proton pump inhibitors (PPIs), microscopic colitis and diverticular disease may also lead to elevated calprotectin level. Patients affected by untreated celiac disease may occasionally show elevated calprotectin values.[5]
- Other intestinal diseases, including many gastrointestinal infections and colorectal cancer, can result in elevated levels of calprotectin. These specimens may test positive with the ALPCO Calprotectin Chemiluminescence ELISA. 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 neutrophilic presence in the stool and is not specific for IBD.
- The manual weighing extraction procedure is to be used for liquid stool samples. The performance characteristics for the ALPCO Easy Stool Extraction Device have not been established for liquid stool samples.

Expected Values

To verify the low clinical cut-off value (50 µg/g), normal stool samples were obtained from asymptomatic individuals with no abdominal complaints and no history of IBS, IBD or other chronic intestinal disorders; this study cohort was separate from those used to establish estimates of the clinical performance of the test device. The expected result for the "normal"/asymptomatic population is < 50 µg/g (normal).

Calprotectin levels were analyzed using the ALPCO Calprotectin Chemiluminescence ELISA on 131 samples obtained from apparently healthy individuals with the following demographics:

- Gender: 65 females / 66 males
- Age: 22 to 82 years old, mean age 42.4 years

With a cut-off of 50 µg/g, 130/131 of the samples were normal/negative with the ALPCO Calprotectin Chemiluminescence ELISA. Values ranged from <7.9 µg/g to 75.1 µg/g, with 86/131 samples measuring below the lower end of the AMR. The 90% confidence intervals for the lower and upper 95% reference limits of the 131 healthy individuals were determined by the non-parametric quantile method, (N+1)p in accordance with CLSI EP28-A3c, Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory, 3rd Edition using Analyse-it Method Validation Edition. The results are as follows:

Lower Limit (90% CI): 0.7 µg/g (0.5 – 0.9 µg/g)

Upper Limit (90% CI): 37.7 µg/g (30.9 – 75.1 µg/g)

Performance Characteristics and Clinical Studies

Summary of Clinical Studies

The estimates of clinical sensitivity, clinical specificity, positive predictive value (PPV), and negative predictive value (NPV) of the ALPCO Calprotectin Chemiluminescence ELISA were determined by comparing analytical test results of the prospectively collected stool specimens against the clinical diagnosis made by the clinical investigator/gastroenterologist (reference standard):

- IBD diagnosis was based on endoscopy results and/or histology of biopsies taken during the endoscopy.

- IBS diagnosis was based on the Rome IV criteria and confirmed by negative endoscopy including the colon and terminal ileum.
- Subjects were diagnosed with "Other GI conditions" when they did not meet the diagnostic criteria for IBD and IBS (Rome IV).

Clinical Diagnosis	Number of Subjects
IBD	76
Ulcerative Colitis (UC)	34
Crohn's Disease (CD)	30
Indeterminant/Undefined	12
IBS	122
Other GI conditions	226
Total	424

	Number of Results in ALPCO Calprotectin Chemiluminescence ELISA Range			
Clinical Diagnosis	**<50 µg/g**	**50 – 100 µg/g**	**>100 µg/g**	**Total**
IBD	6	20	50	76
IBS	116	4	2	122
GI Other	206	9	11	226

Estimates of sensitivity, specificity, PPV, and NPV, along with 95% confidence intervals (Wilson score method for sensitivity/specificity and Mercaldo-Wald logit method for predictive value) were calculated for the ALPCO Calprotectin Chemiluminescence ELISA as an aid in the diagnosis of IBD (n = 424). The estimates of sensitivity, specificity, PPV, and NPV were calculated considering equivocal results as both positive and negative:

Equivocal results = positive	**ALPCO Test Result**		
Clinical Diagnosis	> 50	<= 50	Total
IBD	70	6	76
Non-IBD	26	322	348
Total	96	328	424
	Fraction	**%**	**95% CI**
Sensitivity	70/76	92.1	83.8 – 96.3
Specificity	322/348	92.5	89.3 – 94.9
PPV	70/96	72.9	64.9 – 79.7
NPV	322/328	98.2	96.1 – 99.1

Equivocal results = negative	**ALPCO Test Result**		
Clinical Diagnosis	> 100	<= 100	Total
IBD	50	26	76
Non-IBD	13	335	348
Total	63	361	424

	Fraction	**%**	**95% CI**
Sensitivity	50/76	65.8	54.6 – 75.5
Specificity	335/348	96.3	93.7 – 97.8
PPV	50/63	79.4	68.8 – 87.0
NPV	335/361	92.8	90.4 – 94.6

Estimates of sensitivity, specificity, PPV, and NPV, along with 95% confidence intervals (Wilson score method for sensitivity/specificity and Mercaldo-Wald logit method for predictive value) were calculated for the ALPCO Calprotectin Chemiluminescence ELISA as an aid in the differentiation of IBD vs IBS (n = 198). The estimates of sensitivity, specificity, PPV, and NPV were calculated considering equivocal results as both positive and negative:

Equivocal results = positive	**ALPCO Test Result**		
Clinical Diagnosis	> 50	<= 50	Total
IBD	70	6	76
IBS	6	116	122
Total	76	122	198
	Fraction	**%**	**95% CI**
Sensitivity	70/76	92.1	83.8 – 96.3
Specificity	116/122	95.1	89.7 – 97.7
PPV	70/76	92.1	84.2 – 96.2
NPV	116/122	95.1	90.0 – 97.7

Equivocal results = negative	**ALPCO Test Result**		
Clinical Diagnosis	> 100	<= 100	Total
IBD	50	26	76
IBS	2	120	122
Total	52	146	198
	Fraction	**%**	**95% CI**
Sensitivity	50/76	65.8	54.6 – 75.5
Specificity	120/122	98.4	94.2 – 99.5
PPV	50/52	96.2	86.2 – 99.0
NPV	120/146	82.2	77.1 – 86.3

Method Comparison

A method comparison study was performed comparing the ALPCO Calprotectin Chemiluminescence ELISA and the predicate device. 400 samples that were used to determine the clinical performance characteristics of the ALPCO Calprotectin

Chemiluminescence ELISA were tested on the predicate device according to the manufacturer-supplied labeling.

A qualitative agreement analysis between the ALPCO Calprotectin Chemiluminescence ELISA and the predicate device was conducted including all samples that were tested on both devices (N=400) using the cut-offs of both products to calculate the positive percent agreement (PPA), negative percent agreement (NPA), and total percent agreement (TPA), and 95% CIs (Wilson score method) thereof considering equivocal results as both positive and negative. The results are as follows:

	ALPCO Calprotectin Chemiluminescence ELISA			
Predicate	Normal (< 50 µg/g)	Equivocal (50 – 100 µg/g)	Abnormal (> 100 µg/g)	Total
Normal (< 50 mg/kg)	229	5	12	246
Equivocal (50 – 120 mg/kg)	60	11	9	80
Abnormal (> 120 mg/kg)	22	14	38	74
Total	311	30	59	400

Equivocal results considered positive (95% CI)			
PPA	72/154	46.8%	(39.0 – 54.6%)
NPA	229/246	93.1%	(89.2 – 95.6%)
TPA	301/400	75.3%	(70.8 – 79.2%)
Equivocal results considered negative (95% CI)			
PPA	38/74	51.4%	(40.2 – 62.4%)
NPA	305/326	93.6%	(90.4 – 95.7%)
TPA	343/400	85.8%	(82.0 – 88.8%)

An analytical method comparison was conducted including the samples that were within the analytical measuring range of both assays (N=169); results had to measure within 27.1 – 3000 mg/kg on the predicate device and 7.9 – 6000 µg/g on the ALPCO Calprotectin Chemiluminescence ELISA. A scatter plot was created by plotting the values obtained with the ALPCO Calprotectin Chemiluminescence ELISA (Y-axis) against the predicate device (X-axis) and analyzed by Passing-Bablok regression analysis using Analyse-it Method Validation Edition. The slope, y-intercept, and correlation-r were determined. The 95% CI of the slope and intercept was determined using the bootstrap technique. The results are as follows:

Analytical Method Comparison with Predicate Device	
N	169
Slope (95% CI)	0.6919 (0.5760 – 0.8777)
Y-Intercept (95% CI)	-18.35 (-29.54 - -11.13)
Correlation-r	0.784

Performance characteristics

Precision

The precision of the ALPCO Calprotectin Chemiluminescence ELISA was evaluated internally at ALPCO in accordance with CLSI EP05-A3, Evaluation of Precision of Quantitative Measurement Procedures. The study was performed using one (1) kit lot of ALPCO Calprotectin Chemiluminescence ELISA using one (1) ELISA reader by one (1) operator. Eight (8) stool samples containing various fecal calprotectin concentrations covering a significant portion of the reportable range of the ALPCO Calprotectin Chemiluminescence ELISA were extracted using the Easy Extraction method and the resultant extracts were analyzed in duplicate, twice per day, for 20 days to generate a total of 80 replicates per sample. Data were analyzed using Analyse-it Method Validation Edition. The results are as follows:

			Within-run		Between-run		Within-Day		Between-day		Total	
Sample	N	Mean (µg/g)	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV
1	80	32.9	1.3	3.9%	1.2	3.7%	1.8	5.3%	2.9	8.9%	3.4	10.3%
2	80	39.1	1.8	4.7%	1.6	4.0%	2.4	6.1%	2.8	7.2%	3.7	9.5%
3	80	82.2	3.7	4.5%	3.3	4.0%	4.9	6.0%	3.8	4.6%	6.2	7.6%
4	80	116.6	3.0	2.6%	4.3	3.6%	5.2	4.5%	4.2	3.6%	6.7	5.7%
5	80	288.7	9.5	3.3%	12.3	4.2%	15.5	5.4%	8.6	3.0%	17.7	6.1%
6	80	680.3	19.7	2.9%	26.2	3.8%	32.8	4.8%	29.2	4.3%	43.9	6.4%
7	80	908.3	25.7	2.8%	29.2	3.2%	38.9	4.3%	56.6	6.2%	68.7	7.6%
8	80	5323.5	220.0	4.1%	359.8	6.8%	421.7	7.9%	410.8	7.7%	588.8	11.1%

Lot-to-Lot Reproducibility

The lot-to-lot reproducibility of the ALPCO Calprotectin Chemiluminescence ELISA was evaluated internally at ALPCO in accordance with CLSI EP05-A3, Evaluation of Precision of Quantitative Measurement Procedures. The study was performed using three (3) kit lots of ALPCO Calprotectin Chemiluminescence ELISA using one (1) ELISA reader by one (1) operator. Seven (7) stool samples containing various fecal calprotectin concentrations covering a fraction of the reportable range of the ALPCO Calprotectin Chemiluminescence ELISA were extracted using the Easy Extraction method and analyzed in replicates of 5, once per day, for 5 days using each of the kit lots to generate a total of 75 replicates per sample. Data were analyzed using Analyse-it Method Validation Edition. The results are as follows:

			Within-run		Between-day		Within-lot		Between-lot		Total	
Sample	N	Mean (µg/g)	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV
1	75	35.4	1.8	5.2%	2.1	6.0%	2.8	8.0%	1.8	5.2%	3.4	9.5%
2	75	76.7	3.4	4.4%	4.5	5.9%	5.6	7.3%	1.7	2.2%	5.9	7.7%
3	75	110.8	7.7	6.9%	7.3	6.6%	10.6	9.5%	7.2	6.5%	12.8	11.5%
4	75	288.6	8.5	2.9%	16.5	5.7%	18.5	6.4%	17.2	5.9%	25.3	8.8%
5	75	684.2	23.3	3.4%	45.1	6.6%	50.8	7.4%	49.6	7.2%	71.0	10.4%
6	75	919.2	38.1	4.1%	72.3	7.9%	81.7	8.9%	60.8	6.6%	101.9	11.1%
7	75	4480.2	321.3	7.2%	445.1	9.9%	548.9	12.3%	298.0	6.7%	624.6	13.9%

Site-to-Site Reproducibility

The site-to-site reproducibility of the ALPCO Calprotectin Chemiluminescence ELISA was evaluated internally at ALPCO and at two other analytical test sites in accordance with CLSI EP05-A3, Evaluation of Precision of Quantitative Measurement Procedures. The study was performed using one (1) kit lot of ALPCO Calprotectin Chemiluminescence ELISA by one (1) operator per test site. Seven (7) stool samples containing various fecal calprotectin concentrations of the reportable range of the ALPCO Calprotectin Chemiluminescence ELISA were extracted using the Easy Extraction method at ALPCO, frozen, and then shipped to the two additional sites. Each extract was analyzed in replicates of 5, once per day, for 5 days at each site to generate 25 replicates per site and a total of 75 replicates per sample. Data were analyzed using Analyse-it Method Validation Edition. The results are as follows:

			Within-run		Between-day		Within-site		Between-site		Total	
Sample	N	Mean (µg/g)	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV
1	75	20.0	2.4	12.1%	1.7	8.6%	3.0	14.9%	0.3	1.4%	3.0	15.0%
2	75	21.1	2.2	10.4%	1.7	7.9%	2.8	13.1%	0.0	0.0%	2.8	13.1%
3	75	46.3	2.6	5.6%	3.7	8.1%	4.5	9.8%	0.7	1.6%	4.6	9.9%
4	75	138.2	10.1	7.3%	15.8	11.4%	18.7	13.5%	0.0	0.0%	18.7	13.5%
5	75	195.2	8.9	4.6%	21.6	11.0%	23.3	12.0%	0.0	0.0%	23.3	12.0%
6	75	446.5	21.5	4.8%	27.6	6.2%	35.0	7.8%	0.0	0.0%	35.0	7.8%
7	75	2353.7	125.3	5.3%	172.9	7.3%	213.5	9.1%	137.1	5.8%	253.7	10.8%

Operator-to-Operator Reproducibility

The operator-to-operator reproducibility of the ALPCO Calprotectin Chemiluminescence ELISA was evaluated internally at ALPCO in accordance with CLSI EP05-A3, Evaluation of Precision of Quantitative Measurement Procedures. The study was performed using one (1) kit lot of ALPCO Calprotectin Chemiluminescence ELISA by three (3) operators. Seven (7) stool samples containing various fecal calprotectin concentrations covering a significant portion of the reportable range of the ALPCO Calprotectin Chemiluminescence ELISA were extracted using the Easy Extraction method and analyzed by each operator independently in replicates of 5, once per day, for 5 days to generate 25 replicates per sample, per operator and a total of 75 replicates per sample. Data were analyzed using Analyse-it Method Validation Edition. The results are as follows:

			Within-run		Between-run		Within-operator		Between-operator		Total	
Sample	N	Mean (µg/g)	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV
1	75	26.7	0.9	3.5%	2.1	8.0%	2.3	8.7%	2.3	8.6%	3.3	12.3%
2	75	32.5	1.6	5.0%	3.2	9.7%	3.6	10.9%	3.6	11.0%	5.0	15.5%
3	75	68.1	2.1	3.1%	5.8	8.5%	6.1	9.0%	8.4	12.3%	10.4	15.3%
4	75	98.8	3.9	3.9%	4.8	4.8%	6.1	6.2%	5.8	5.8%	8.4	8.5%
5	75	555.8	22.2	4.0%	26.2	4.7%	34.4	6.2%	70.9	12.8%	78.8	14.2%
6	75	765.0	29.5	3.9%	81.3	10.6%	86.5	11.3%	42.5	5.6%	96.4	12.6%
7	75	4468.6	299.8	6.7%	470.4	10.5%	557.9	12.5%	225.5	5.0%	601.7	13.5%

Extraction Method Reproducibility

The extraction reproducibility of the ALPCO Calprotectin Chemiluminescence ELISA was evaluated internally at ALPCO. The study was performed using one (1) kit lot of ALPCO Calprotectin Chemiluminescence ELISA by one (1) operator. Sets of seven (7) stool samples containing various fecal calprotectin concentrations, including stool samples that are near the clinical cut-offs of the ALPCO Calprotectin Chemiluminescence ELISA were extracted 10 times using both the Easy Extraction method and manual weighing method. Each stool sample extract was analyzed in duplicate to generate 20 replicates per sample, per extraction method. Data were analyzed using Analyse-it Method Validation Edition. The results are as follows:

Easy Extraction method reproducibility using the Easy Extraction Device:

			Within-Extraction		Between Extraction		Total Imprecision	
Sample	N	Mean (µg/g)	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV
1	20	33.4	1.7	5.1%	0.0	0.0%	1.7	5.1%
2	20	45.9	4.6	9.9%	2.8	6.1%	5.3	11.6%
3	20	68.3	3.5	5.1%	3.2	4.7%	4.8	7.0%
4	20	123.7	6.9	5.6%	3.9	3.1%	7.9	6.4%
5	20	129.0	1.8	1.4%	6.2	4.8%	6.5	5.0%
6	20	1748.5	186.5	10.7%	121.4	6.9%	222.6	12.7%
7	20	4298.5	317.2	7.4%	426.9	9.9%	531.8	12.4%

Extraction reproducibility using the manual weighing method:

			Within-Extraction		Between Extraction		Total Imprecision	
Sample	N	Mean (µg/g)	SD (µg/g)	%CV	SD (µg/g)	%CV	SD (µg/g)	%CV
1	20	25.7	1.1	4.5%	1.6	6.4%	2.0	7.8%
2	20	69.6	3.2	4.6%	0.3	0.5%	3.2	4.6%
3	20	103.7	2.5	2.4%	3.7	3.6%	4.5	4.3%
4	20	122.0	5.1	4.1%	8.9	7.3%	10.2	8.4%
5	20	298.6	13.5	4.5%	26.2	8.8%	29.5	9.9%
6	20	1336.1	42.4	3.2%	113.5	8.5%	121.2	9.1%
7	20	4063.3	389.2	9.6%	0.0	0.0%	389.2	9.6%

Matrix and Aqueous Linearity

The matrix linearity and aqueous linearity of the analytical measuring range of the ALPCO Calprotectin Chemiluminescence ELISA were evaluated internally at ALPCO in accordance with CLSI EP06-A, Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach. To evaluate matrix linearity, a stool sample extract containing a high concentration of fecal calprotectin was serially diluted 1:2 with a stool sample extract containing a low concentration of fecal calprotectin to obtain dilution levels with values that cover the entire AMR. Stool sample extracts were obtained using the Easy Extraction method. Each stool sample extract combination was assayed in duplicate in a single analysis using one reagent lot of the ALPCO Calprotectin Chemiluminescence ELISA. To evaluate aqueous linearity, a sample made by diluting native antigen in standard diluent buffer containing stabilizers and preservatives (the same buffer used to make the controls and calibrators) was serially diluted 1:2 with standard diluent buffer to obtain dilution levels with values that cover the entire AMR. Each aqueous sample dilution was assayed in duplicate in a single analysis using one reagent lot of the ALPCO Calprotectin Chemiluminescence ELISA. Results were analyzed using Analyse-it Method Validation Edition to determine the best fitting polynomial. For linearity to be claimed, the best fitting polynomial had to be linear (first order) or the difference between the best fitting nonlinear polynomial (second or third order) and the linear polynomial could not

exceed ±15%. The plots were further analyzed by linear regression. The slope and y-intercept, and the 95% confidence intervals thereof, and R^2 of the regression analysis were calculated. In addition, the recovery of each replicate value included in the regression analysis for the linearity dilutions was calculated and the average recovery was calculated. Acceptance criteria were met, the ALPCO Calprotectin Chemiluminescence ELISA is acceptably linear over the analytical measuring range. The results are as follows:

Sample Type	Test Range (µg/g)	Slope (95% CI)	Y-Intercept (95% CI)	R^2	Average Recovery (%)
Matrix	2.5 – 5780.1	1.003 (0.994 to 1.013)	1.77 (-15.87 to 19.42)	1.00	99.2
Aqueous	3.0 – 6221.7	0.99 (0.98 to 1.00)	0.03 (-22.42 to 22.47)	0.99	100.6

Limits of Blank, Detection and Quantitation

Limit of Blank (LoB)

The limit of blank of the ALPCO Calprotectin Chemiluminescence ELISA was determined in accordance with CLSI EP17-A2. Four blank stool samples were extracted and tested in replicates of six on two reagent lots across three days. The LoB was determined to be 3.6 µg/g by the nonparametric method.

Limit of Detection (LoD)

The limit of detection of the ALPCO Calprotectin Chemiluminescence ELISA was determined in accordance with CLSI EP17-A2. Four stool samples containing a low level of calprotectin were extracted and tested in replicates of five on two reagent lots across three days. The LoD was determined to be 7.7 µg/g by the parametric method.

Limit of Quantitation (LoQ)

The limit of quantitation of the ALPCO Calprotectin Chemiluminescence ELISA was determined in accordance with CLSI EP17-A2. Six stool samples containing a low level of calprotectin were extracted and tested in replicates of five on two reagent lots across three days. The mean and within laboratory precision were calculated for each sample for each reagent lot. For each reagent lot, the observed precision (%CV) (Y-axis) was plotted vs. the sample calprotectin concentration (X-axis) to give a precision profile, and fit by a constant variance function using Analyse-it Method Validation Edition. The LoQ estimate for each reagent lot was determined as the measurand concentration at the intersection of the precision profile curve with the accuracy goal of 20 %CV. The LoQ was determined to be 7.9 µg/g.

Accuracy/Recovery

The accuracy/recovery of the ALPCO Calprotectin Chemiluminescence ELISA was evaluated using seven (7) extracted stool samples containing various concentrations of calprotectin across the analytical measuring range of the assay, samples were extracted using the Easy Extraction method. Native calprotectin diluted in standard diluent was used as the spiking material. The stool sample extracts were mixed with the spiking material in a proportion of 9:1 (9 parts sample:1 part spiking material) to calculate recovery: Samples with calprotectin concentrations lower than 200 µg/g were spiked with 42.8 µg/g. Samples with calprotectin concentrations higher than 200 µg/g were spiked with 129.3 µg/g. Each baseline sample extract, control spike and spiked sample were tested in duplicate in the same assay run. The results are as follows:

Sample	Mean Baseline Result (µg/g)	Spike Value (µg/g)	Theoretical Post-Spike Result (µg/g)	Observed Post-Spike Result (µg/g)	Recovery (%)
1	28.1	42.8	68.1	72.1	105.9
2	36.2	42.8	75.3	79.0	104.9
3	77.7	42.8	112.7	123.6	109.7
4	94.8	42.8	128.1	123.5	96.4
5	248.5	129.3	353.0	391.0	110.8
6	615.5	129.3	683.2	754.1	110.4
7	5392.3	129.3	4982.4	4469.3	89.7

Stool Sample Extraction Method Comparison

The extraction methods of the ALPCO Calprotectin Chemiluminescence ELISA were evaluated internally at ALPCO to determine if the extraction methods provide similar results. The study was performed using one (1) kit lot of ALPCO Calprotectin Chemiluminescence ELISA by one (1) operator. Sixty-eight (68) stool samples varying in consistency and containing various fecal calprotectin concentrations over a significant fraction of reportable range and near the clinical cut-offs of the ALPCO Calprotectin Chemiluminescence ELISA were extracted in parallel using both the Easy Extraction method and manual weighing method. Each resultant stool sample extract was analyzed in singlicate. 61 of the samples were within the AMR of the ALPCO Calprotectin Chemiluminescence ELISA and included in the analysis. A scatter plot was created by plotting the values obtained from the Easy Extraction method extracts (Y-axis) against the manual weighing method extracts (X-axis) and analyzed by Passing-Bablok regression analysis using Analyse-it Method Validation Edition. The Y-Intercept, slope, and bootstrap 95% confidence intervals thereof, and correlation r were calculated. The results are as follows:

Stool Sample Extraction Analytical Method Comparison	
N	61
Slope (95% CI)	1.014 (1.004 to 1.031)
Y-Intercept (95% CI)	-1.296 (-1.986 to -0.3179)
Correlation r	0.997

Qualitative agreement analysis between the Easy Extraction method and manual weighing method was also conducted to calculate the positive percent agreement, negative percent agreement, and total percent agreement and Wilson 95% CI considering equivocal results as both positive and negative. The results are as follows:

Stool Sample Extraction Qualitative Method Comparison

		Manual weighing method			Totals
		Positive	Equivocal	Negative	
Easy Extraction method	Positive	25	0	0	25
	Equivocal	1	13	0	14
	Negative	0	0	22	22
	Totals	26	13	22	61
	Equivocal results considered positive (95% CI)				
	PPA	39/39	100%	(91.0 – 100%)	
	NPA	22/22	100%	(85.1 – 100%)	
	TPA	61/61	100%	(94.1 – 100%)	
	Equivocal results considered negative (95% CI)				
	PPA	25/26	96.2%	(81.1 – 99.3%)	
	NPA	35/35	100%	(90.1 – 100%)	
	TPA	60/61	98.4%	(91.3 – 99.7%)	

References:

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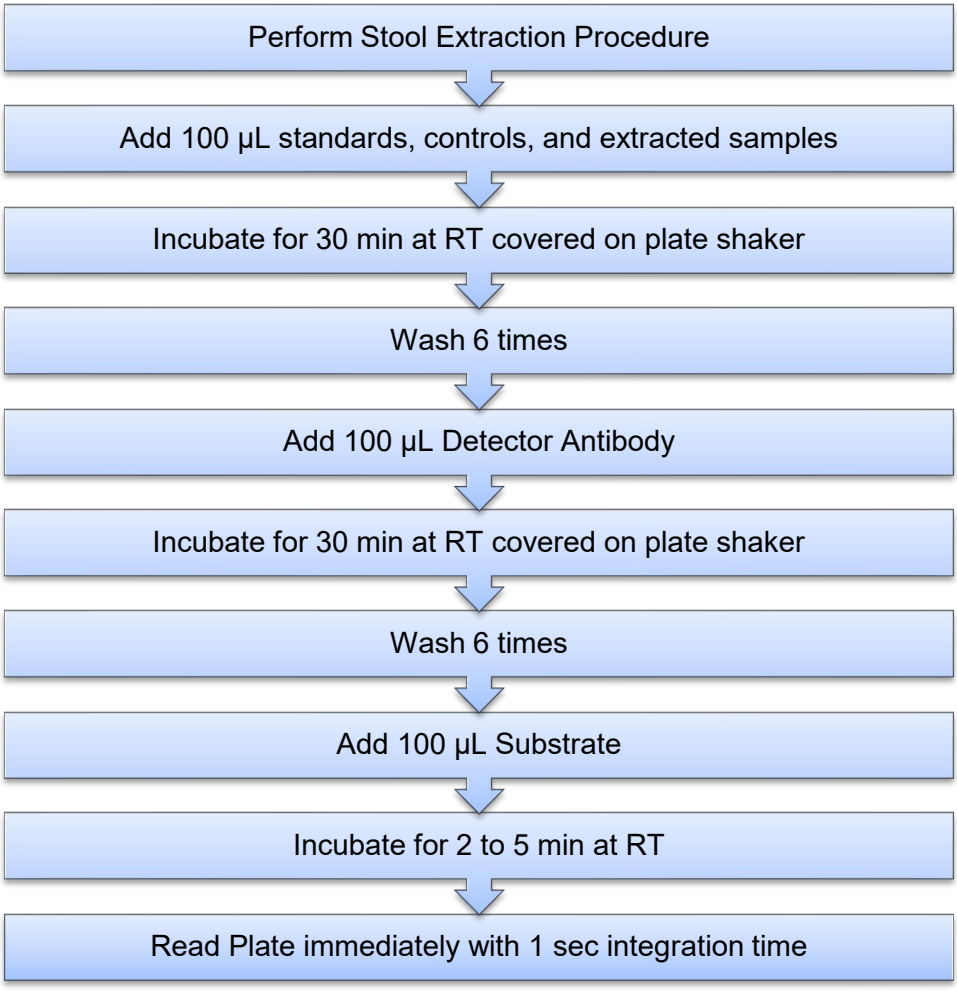
Revision 6.0

SHORT ASSAY PROTOCOL



Total incubation time = 1 h 2 min