PhiCal™ Test

PhiCal Test
Fecal Calprotectin Immunoassay

Intended use
The PhiCal™ test is a quantitative ELISA for measuring, in human stool, concentrations of fecal calprotectin, a neutrophilic protein that is a marker of mucosal inflammation. The PhiCal™ test can be used as an *in vitro* diagnostic to aid in the diagnosis of inflammatory bowel diseases (IBD): Crohn's disease and ulcerative colitis, and to differentiate IBD from irritable bowel syndrome.

Summary and Explanation
Various types of organic disease in the gastrointestinal tract will cause damage to the intestinal lining (mucosa layer). Such damage may vary from increased permeability of the mucosa to inflammation and ulcerations. The bowel content is rich in bacteria and other microrganisms releasing substances which may be toxic or chemotactic, i.e. they stimulate leukocytes, in particular polymorphonuclear granulocytes (PMNs) to migrate into the gut lumen where they release their contents, including antimicrobial substances like calprotectin. This protein constitutes about 60% of total proteins in the cytoplasm of PMN[1] and can be estimated in small, random stool samples even after storage for seven days at ambient temperature.[2] The concentration of calprotectin in stools reflects the number of PMNs migrating into the gut lumen.[3-4]
Calprotectin is a calcium and zinc binding protein produced by PMNs, monocytes and squamous epithelial cells except those in normal skin. [6-8] After binding calcium it can resist degradation by leukocytic and bacterial enzymes.[2,9] By competing with different enzymes for limited local amounts of zinc, calprotectin may inhibit many zinc dependent enzymes[10] and thereby kill microorganisms or animal and human cells in culture.[11-12]
Calprotectin can be detected even in small (less than one gram) random stool samples.[13] Furthermore, organic diseases of the bowel give a strong fecal calprotectin signal, i.e. elevations are often five to several thousand times the upper reference in healthy individuals[2, 5, 14-15] indicating intestinal inflammation.
Patients with organic or functional abdominal disorders may have similar symptoms, and clinical examination alone may not be sufficient to give a specific diagnosis. Additionally, the PhiCal™ test has been demonstrated to be a marker of inflammatory bowel disease in both children and adult patients.[18,19] Inflammatory bowel disease (IBD), i.e. ulcerative colitis and Crohn's disease, may appear from early childhood to late adulthood, and the diagnosis is often delayed due to vague symptoms or reluctance to perform endoscopy and biopsy.

Principle of the test
The PhiCal™ test fecal calprotectin immunoassay is an enzyme-linked immunosorbent assay system with colorimetric detection. The test uses a polyclonal antibody against calprotectin in an enzyme linked immunosorbent assay system. Calprotectin present in the dilute sample is bound by the antibody adsorbed to the surface of the plastic well. The enzyme conjugated antibody binds to the captured antigen and subsequently the enzyme catalyzes the conversion of the substrate to a colored product. The intensity of the color 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 standards.

Reagents included with the kit
1. **Antibody coated plate:** 12 strips, 8 wells per strip, coated with antibodies against calprotectin. The plate is stored in a sealed bag with desiccant.
2. **Conjugate:** 15 ml alkaline phosphatase labelled, immunoaffinity purified IgG antibodies (from rabbit) against calprotectin in a buffer solution with Proclin 300 as a preservative.

3. **Substrate:** 1 vial containing 15 ml substrate in buffer. Ready to use.
4. **Washing solution:** 50 mL 20X concentrate, to be diluted with distilled water.
5. **Dilution solution:** 20 mL 10X concentrate with Proclin 300 as a preservative, to be diluted with distilled water.
6. **Extraction solution:** 2x50 mL 2.5X concentrate with Proclin 300 as a preservative, to be diluted with distilled water. This concentrated solution is irritating to eyes and skin.
7. **Calibrators:** 5 vials with 1 ml calprotectin solution at known concentrations (6.25, 12.5, 25, 50 and 100 ng/ml) with Proclin 300 as a preservative. The value of each standard is printed on the vial label.
8. **Controls:** With Proclin 300 as a preservative.
   Control 1 (Low): one vial containing 1.0 ml. Ready to use. Do not dilute. The lot-specific range of values is printed on the vial label and is expressed as ng/ml. Approximate range = 20-40 ng/ml or (50-100 µg/g).
   Control 2 (High): one vial containing 1.0 ml. Ready to use. Do not dilute. The lot-specific range of values is printed on the vial label and is expressed as ng/ml. Approximate range = 45-75 ng/ml or (112.5-187.5 µg/g).

Precautions and warnings

For *in vitro* diagnostic use.

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. The substrate reagent contains sodium azide as preservative at concentrations less than 0.1 % (w/w). On disposal, flush with large volumes of water to prevent the buildup of potentially explosive metal azides in lead and copper plumbing.

When handling extraction solution, wear suitable protective clothing. In case of contact with eyes rinse immediately with plenty of water and seek medical advice.

All reagents, except for the substrate and the concentrated washing solution, contain Proclin 300 as a preservative agent below the allowed limits.

Warning: do not interchange components from the different kit batches. Satisfactory performance of the test is guaranteed only when components from the same kit lot are used.

Kits should not be used beyond their expiration date.

Avoid mixing caps between reagent vials.

Keep the bottom surface of wells clean and avoid scratching.

Preparation of working solutions

Extraction solution

Dilute concentrated Extraction solution by adding 1 part (50 ml) of it to 1,5 parts (75 ml) of freshly distilled water to obtain 125 ml working solution. Mix well.

Dilution solution

Dilute concentrated Dilution solution by adding 1 part (20 ml) of it to 9 parts (180 ml) of distilled water to obtain 200 ml working solution. Mix thoroughly.

Washing solution

Dilute concentrated Washing solution by adding the contents of the vial (50 ml) to distilled water to a final volume of 1000 ml.

Storage and stability of reagents and working solutions

1. The expiration date is printed on all component labels. The kit containing the reagents with designated shelf-life should be stored at 2-8°C.
2. Avoid exposure to high temperature, direct sunlight or extreme humidity.
3. Unused microtiter strips should be resealed airtight in the plastic bag with the dessicant inside and stored at 2-8°C. They can be stored for one month.

Reagent stability (unsealed reagents)

Reagent	Storage conditions	Storage time
Conjugate	2-8 °C	1 month
Substrate	2-8 °C	3 months
Calibrators	2-8 °C	1 month
Controls	2-8 °C	1 month

Substrate reagent should be pale yellow. The substrate is light sensitive. Store in the dark and shake before use.

Stability of working solutions

Reagent	Storage conditions	Storage time
Washing solution	20-25 °C	7 days
Extraction solution	2-8 °C	3 months
Dilution solution	2-8 °C	7 days

Specimen collection

Random stool 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.

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

Specimen transport: Stool specimen should be received by the laboratory within 10 days of collection. Temperature during shipment should not exceed 37°C. Sample must be extracted within 11 days of collection.

Storage: 11 days at variable ambient temperature (not to exceed 37°C) is acceptable. Samples should be stored @ 2-8°C for up to 11 days, or for 1 year @ -20°C.

Assay Procedure

Materials provided with the kit

1. Antibody coated plate
2. Conjugate
3. Substrate
4. Washing solution
5. Dilution solution
6. Extraction solution
7. Calibrators
8. Controls

Materials required but not provided

1. Specimen collection container
2. Specimen transport tube
3. Wooden applicator sticks
4. Disposable polystyrene screw-cap tubes, 14 ml
5. Eppendorf tubes (1 - 1.5 ml)
6. Sensitive digital scale (40-150 mg)
7. Vortex mixer
8. Shaker
9. Microcentrifuge (10,000 x g)
10. Freezer (-20°C)
11. Multi-channel pipette, 50-200 µl

12. ELISA plate washer
13. ELISA plate reader (filter 405 nm)
14. Distilled water
15. Stop solution (NaOH 1M) [optional]

Specimen Preparation Procedure

1. Thaw frozen stool samples at room temperature and ensure that all reagents reach room temperature (20-25°C). Recommended batch sizes are 10, 20, 30, or 40 samples.
2. Weigh (tare) the empty screw cap tube.
3. Using the wooden applicator stick, transfer approximately 100 mg (between 80 - 120 mg) feces and place into a screw-cap tube.
4. Weigh tube with feces. Net feces weight should be between 80 - 120 mg.
5. Break off a clean wooden applicator stick inside the screw-cap tube, for use as an agitator.
6. Add diluted extraction solution to the screw-cap tube such that the weight/volume ratio is 1:50 (e.g. 100 mg feces + 4.9 ml diluted extraction solution). The table below provides the volume of diluted extraction solution to be added to a given amount of stool.

Stool (mg)	Diluted extraction solution (ml)
120	5.9
115	5.6
110	5.4
105	5.2
100	4.9
95	4.7
90	4.4
85	4.2
80	3.9

7. Close the tube. Shake or mix vigorously for 30 seconds using a vortex mixer. Mixing time should be as accurate as possible.
8. Homogenize 25 ± 5 minutes on a shaker or roller. The specimen should not be homogenized for less than 20 minutes. Reduce delay before pipetting.
9. Immediately transfer 1 ml of the homogenate to an Eppendorf tube and centrifuge for 20 minutes at 10,000 x g at room temperature using a bench-top centrifuge (e.g. Heraeus Biofuge 13). Do not change centrifugation time and g force. Check g force and rpm. For ease of harvesting and handling, make sure the pellet is compact at the bottom of the centrifuge tube.
10. Transfer 0.5 ml of the top half of the clear extract supernatant to a new Eppendorf tube. Avoid contact with the pellet as aggregates or particles can cause erroneous calprotectin values.
11. The extracts may be tested immediately. Extracts may be stored at -20°C for up to 3 months for later measurement.

ELISA Procedure

Procedural Notes

- *Ensure that all reagents, samples and antibody coated strips reach room temperature (20-25°C) before starting the test.*
- *Thaw frozen sample extracts at room temperature.*
- *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.*
- *Avoid carry-over from conjugate to substrate if only one pipette is used. Avoid the formation of bubbles during substrate pipetting. Use separate reservoirs for conjugate and substrate if different than originals vials.*

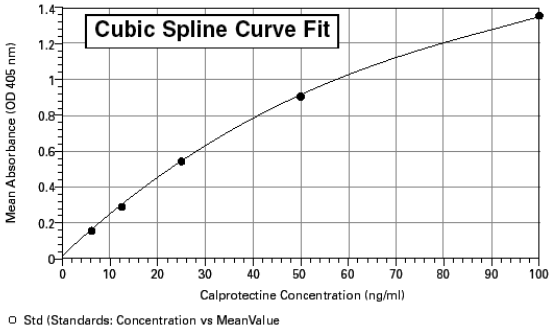
1. Add 20 µl of sample extract to 980 µl of working dilution solution (1:50 dilution). For further dilution of high concentration samples, dilute 200 µl of the 1:50 dilution of the sample extract with 800 µl of working dilution solution for a final 1: 250 dilution.
2. Suggested plate layout in duplicates 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. Blank, standards and controls must be included in each run.

	1	2	3	4	5	6
A	Blank	Calibrator 4	Sample 1	Sample 5	Sample 9	Sample 13
B	Blank	Calibrator 4	Sample 1	Sample 5	Sample 9	Sample 13
C	Calibrator 1	Calibrator 5	Sample 2	Sample 6	Sample 10	Sample 14
D	Calibrator 1	Calibrator 5	Sample 2	Sample 6	Sample 10	Sample 14
E	Calibrator 2	Control 1	Sample 3	Sample 7	Sample 11	
F	Calibrator 2	Control 1	Sample 3	Sample 7	Sample 11	
G	Calibrator 3	Control 2	Sample 4	Sample 8	Sample 12	
H	Calibrator 3	Control 2	Sample 4	Sample 8	Sample 12	

3. Add 100 µl of working dilution solution to wells A1-B1 (blank).
4. Add 100 µl of each calibrator in duplicate wells (C1-D1, E1-F1, G1-H1, A2-B2, C2-D2).
5. Add 100 µl of each control in duplicate wells (E2-F2, G2-H2).
6. Mix diluted sample well before application to the plate and add 100 µl of each sample in duplicate wells (A3-B3, C3-D3, etc.).
7. Cover plate with plate cover and incubate at room temperature for 45 ± 5 min.
8. At the end of the incubation time, wash the plate by adding 0.4 ml of diluted washing solution to each well. Repeat this step two more times up to a total of 3 washing steps. Avoid blocking of aspiration or filling probes. After the final aspiration, invert plate and tap gently on absorbent tissue to ensure complete removal of washing solution. Remove as much liquid as possible.
9. Mix conjugate prior to use (do not shake). Add 100 µl conjugate to each well.
10. Cover plate with plate cover and incubate the plate at room temperature for 45 ± 5 min.
11. Repeat washing step as above (see step 8).
12. Add 100 µl of substrate solution to each well. A multi-channel pipette is recommended in order to avoid variation in substrate development time.
13. Incubate the plate at room temperature for approximately 30 minutes in a dark place or wrap the plate with aluminum foil.
14. Read the optical density (OD) values by means of an ELISA reader at 405 nm. When calibrator 5 reaches an OD value between 1.2-1.5, the reaction should be read with an automatic EIA reader or stopped by adding 100 µl of 1M NaOH stop solution. Plates stopped with 1M NaOH may be stored at 4°C for 24 hours.

Calprotectin Quantitation

A new standard curve is determined for each run using the five calibrators provided in the kit
Calibrator S1: 6.25 ng/ml calprotectin
Calibrator S2: 12.5 ng/ml calprotectin
Calibrator S3: 25 ng/ml calprotectin
Calibrator S4: 50 ng/ml calprotectin
Calibrator S5: 100 ng/ml calprotectin
Calculate the mean OD of the duplicates of the calibrators and the blank. Subtract the OD value of the blank from all values. Plot the standard curve with the calprotectin concentration in ng/ml for each calibrator (x-axis) and the corresponding mean OD value (y-axis). See example of standard curve below:



Assay quality control

1. The plate background should be < 0.15 OD (average of blanks).
2. Controls 1 and 2 are to be included in each run. Control values must fall within the manufacturer-defined lot-specific ranges noted on each vial for run to be valid.

Reportable Range

6.25 - 100 ng/ml (15.625 - 250 µg/g)

Calculation of sample test results

Calculate the mean OD of the duplicates for each sample. Subtract the OD value of the blank from all values. Determine the concentration of calprotectin as detailed below. If a sample required additional dilution, this must be accounted for during calculations. Samples with results between 50-120 µg/g are recommended for re-evaluation in 4-6 weeks.

Determination of calprotectin levels in patient samples requires a calculation involving dilution correction and stool weight normalization. The following data is required for calculation of calprotectin in the units of microgram/gram of stool.

Raw Stool Weight (CALSW) (Actual amount used for extraction.) (Acceptable range = 0.080 to 0.120 grams)	**X (g)**
Calprotectin ELISA Result (CALPR) (Reportable Range 6.25 to 100 ng/ml)	**X (ng/mL)**
Dilution Factor (CALDF) (Standard dilution is 50) (Additional dilution factors for values >250 µg/g may be incorporated)	**50**
Extraction Buffer Volume (EXBV)	**5(mL)**

Example:

ELISA Result (CALPR) = 40 ng/ml,
Raw Stool Weight (CALSW) = 0.1000 g

Calculation: [(40 ng/ml * 50 * 5 mL) / 0.1000 g] / 1000 = 100.0 µg/g

CALPR*CALDF*EXBV CALSW Unit Conversion ng/g to µg/g

Limitations

- False-negative results could occur in patients who have granulocytopenia due to bone marrow depression.
- Some patients who are taking NSAIDs will have elevations in their fecal calprotectin levels.[18,20]
- Results may not be clinically applicable to children younger than 2 y.o., who have mildly increased calprotectin levels.[21]
- Patients with IBD fluctuate between active (inflammatory) and inactive stages of the disease. These stages must be considered when using the PhiCal™ Test.
- Other intestinal ailments, including many gastrointestinal infections and colorectal cancer, can result in elevated levels of calprotectin. These specimens will test positive with the PhiCal™ Test. Therefore, a diagnosis of active IBD cannot be established solely on the basis of a positive result with the PhiCal™ Test."
- Fecal calprotectin is an indicator of neutrophilic presence in the stool and is not specific for IBD.

Expected values

Calprotectin Concentration	Interpretation	Follow-Up
<15.625 - 50 µg/g	Normal	None
>50 - 120 µg/g	Borderline	Re-evaluate after 4-6 weeks
>120 µg/g	Abnormal	Repeat as clinically indicated

Initial cut-off of a normal healthy reference range was performed by Roseth *et al.* (1992). Further evaluation to include asymptomatic patients, as well as patients with IBS (to differentiate from IBD) were additionally performed. Data analysis demonstrates that in multiple cohorts of asymptomatic patients and those with IBS, the fecal calprotectin level shows:

Median value = 20-24 Mean value = 40 Std Dev = 40

Thus, the mean +/- 2SD gives an upper value of 120.

Clinical Studies

Clinical studies gave the following values:

Study cohort	PhiCal™ Value*	Range	n-value	Ref #
Normal healthy reference	12.5 µg/g*	2.5 - 54	48	2
Normal healthy reference	11 µg/g	CI 3 - 18	34	17
Normal healthy reference	9.3 µg/g	6.5 - 63	27	22
IBD - Crohn's & Colitis, NOS	220 µg/g*	20 - 9000	38	2
Ulcerative Colitis (low/no activity)	60 µg/g*	25 - 125	36	15
Ulcerative Colitis (active disease)	340 µg/g*	195 - 655	28	15
Ulcerative Colitis (Dx)	167 µg/g	CI 59 - 276	82	17
Ulcerative Colitis (Dx)	290 µg/g*	140 - 620	37	19
Crohn's Disease	455 µg/g*	210 - 5000+	119	4
Crohn's Disease (Dx)	231 µg/g	CI 110 - 353	49	17
Crohn's Disease (Dx)	475 µg/g*	205 - 710	43	19
Crohn's Disease	230 µg/g	6.5 - 11966	25	22
Irritable Bowel Syndrome	20 µg/g*	2.5 - 250	159	4
Irritable Bowel Syndrome	20 µg/g*	2.5 - 250	339	16
Irritable Bowel Syndrome	22 µg/g	CI 9 - 35	48	17
Irritable Bowel Syndrome	20.5 µg/g	6.5 - 87	25	22

*Median values adjusted for 5x change in extraction.

Comparison Studies

64 patients undergoing endoscopy were evaluated with lactoferrin and calprotectin. 53 patients were included sequentially among those attending the Department of Gastroenterology, Aker University Hospital, Oslo, Norway for check-up of their IBD. Eleven patients were healthy, normal patients presenting for routine colonoscopic screening. Fecal calprotectin and lactoferrin levels were assessed. Evaluation of disease activity was based upon endoscopic change.

n = 64	Healthy	Inactive IBD	Low IBD	High IBD
Lactoferrin (+)	0	0	3	18
Lactoferrin (-)	11	19	3	18

PhiCal >120	0	1	6	28
PhiCal 50-120	1	6	0	0
PhiCal <50	10	12	0	0

Stool results from 908 subjects from the clinical studies table above were used to calculate the clinical sensitivity and specificity of the PhiCal test. The 908 subjects included IBD (n=255), IBS (n=410), other bowel diseases (n=82), and normal subjects (n=161). Of this cohort, 19 subjects were children ranging from 1-12 years of age, 9 of whom had IBD. The results and calculations show:

n = 908	IBD/ Inflamed "Organic Disease"	IBS/ Normal "Non-Organic"	Totals
> 120 µg/g	254	29	283
50-120 µg/g	43	89	132
<50 µg/g	42	451	493
Totals	296 + 43	480 + 89	776 + 132

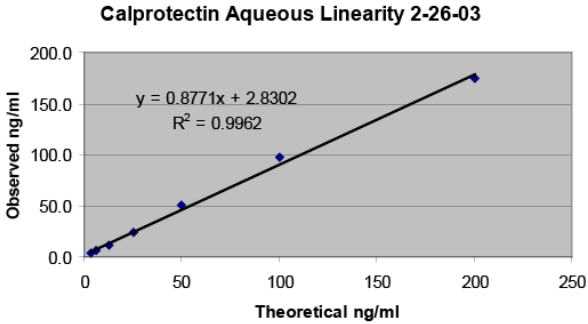
When borderline cases (n=132) are not included in the calculations:

Clinical Sensitivity	**86%** (254/296)
Clinical Specificity	**94%** (451/480)
Agreement	**91%** (705/776)

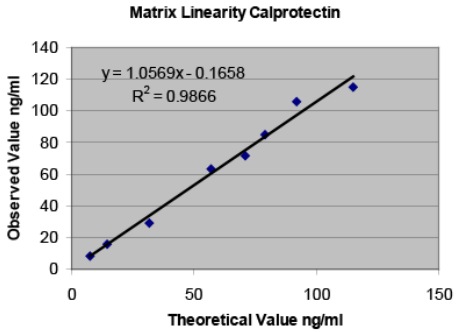
Performance characteristics

Aqueous linearity:

The aqueous linearity was performed by making dilutions of a calprotectin reference material (obtained from Dr. Magne Fagerhol in Oslo, Norway). Triplicate measurements were made for each dilution level. The mean observed value for each level was compared to the corresponding expected values as shown in the graph below. The assessment of aqueous linearity shows that the assay has both acceptable linearity and accuracy from 6.25 - 100 ng/ml or 15.625 to 250 µg/g. This study also serves as a second-source calibration verification, as the standards that were used to calibrate were provided with the kit and that which was used to assess linearity consisted of dilutions of a purified second-source material.



Matrix linearity:
The matrix linearity study was performed by making dilutions of the extract from a stool sample that had previously tested at the high end of the calibration range; dilutions were made using dilution buffer. Each dilution level was assayed in triplicate and the mean observed value for each level was compared to its corresponding theoretical value (calculated by taking the 100% extract solution as the "true"). Results are shown below. The assessment of matrix linearity shows that the assay has both acceptable linearity and accuracy from 7.73 - 114.899 ng/ml or 19.325 - 287.2475 µg/g. However, it was decided to limit the reportable range to the values of the lowest and highest calibrators contained within the assay kit.



Functional Sensitivity
The functional sensitivity (FS), along with linearity data, determine the lowest concentration at which results are both accurate and precise; patient results that fall below the functional sensitivity of the assay are reported as <FS. To establish the FS for the PhiCal™ Test, 3 concentrations of kit standards (12.5 ng/ml, 6.25 ng/ml, 3.125 ng/ml) were assayed in either triplicate or quadruplicate over 6 days; the 3.125 ng/ml level was obtained by diluting the 6.25 ng/ml standard using dilution buffer. The %CV for each level was plotted against its respective concentration. Accuracy and precision are acceptable for the assayed concentrations 3.125 ng/ml, 6.25 ng/ml and 12.5 ng/ml. The reportable range will be limited to the value of the lowest calibrator: 6.25 ng/ml.
6.25 ng/ml correspond to 15.6 (µg calprotectin) / (g stool) at a typical sample dilution of 1:2500.

Accuracy/ Recovery

Extracts from five different stool samples were each spiked with calprotectin. The calprotectin used for the spike was obtained from established serum pools. The baseline extract for each sample was "spiked" with assay buffer to compensate for volume adjustments made to the calprotectin-spiked extracts. Each of the spiked extracts was then assayed per kit protocol. Data is shown in table below.

Calprotectin Recovery Data					
	# 1a	**# 2a**	**# 3a**	**# 4a**	**# 5a**
Baseline (µg/g)	49.1	73.4	66.4	6.4	73.6
Spike Value (µg/g)	58.5	57.6	55.2	55.3	59.0
Theoretical (Base + Spike) (µg/g)	107.6	131.0	121.7	61.7	132.7
Observed (Base + Spike) (µg/g)	106.7	129.9	144.3	66.1	140.0
% Recovery	**99.2**	**99.1**	**118.6**	**107.2**	**105.5**

Precision:

Intra-Assay precision was determined by extracting one low and one high matrix sample, and assaying each extract twenty times within a single assay run. Precision is calculated as the % CV obtained for each level. Respective CV's were 4.07% and 6.17%. Additionally, 12 samples were assayed in 6 replicates within a singly assay run. See table below. The resultant %CVs demonstrate that the PhiCal Test is precise in the reportable range.

Calprotectin Intra-Assay Precision (mg/g)													
Analysis		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	Mean	177.5	55.3	229.4	31.1	96.9	106.8	17.5	18.7	20.8	182.8	55.6	60.2
	Std Dev	5.7	6.6	6.7	2.3	11.5	9.4	0.8	2.7	1.9	10.8	3.9	2.5
	%CV	3.2	12.0	2.9	7.4	11.9	8.8	4.7	14.3	9.2	5.9	7.0	4.1

Inter-Assay precision was determined by extracting and assaying one low and one high matrix sample across 20 individual assay runs. Precision is calculated as the % CV obtained for each level. Respective CV's were 5.66% and 6.78%. Additionally, ten different samples (5 positives and 5 negatives) were extracted 5 separate times from individual stool aliquots. Each extract was then assayed in 4 replicates on 5 separate EIA runs performed on 5 different days. See table below. The resultant %CVs demonstrate that the PhiCal Test is precise in the reportable range.

Calprotectin Inter-Assay Precision (mg/g)											
Analysis	**Replicate**	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**S10**
	Mean	36.9	12.9	16.6	20.2	26.9	131.7	188.2	89.8	101.1	63.3
	Std Dev	2.1	2.1	2.5	0.9	2.0	10.2	36.9	10.4	13.3	12.7
	%CV	5.8	16.7	14.9	4.4	7.6	7.7	19.6	11.6	13.2	20.1

Extraction Reproducibility:
In order to determine how reproducible values were from extraction-to-extraction, two samples that represent the low and high ends of the reportable range were chosen. Each was extracted 24 times, and each extract was tested. Precision was evaluated according to the %CV obtained for each level. Respective CV's of 12.60% and 12.13% for the low and high levels demonstrate that results are reproducible along the reportable range.

Analytical Specificity
Microorganisms
The following bacteria, which occur frequently in the stool, or are common to infectious diarrhea were evaluated as potential interfering factors - *Escherichia coli*, *Citrobacter freundii*, and *Klebsiella pneumoniae, Salmonella, Shigella, Yersinia.* The presence of these bacteria in stool samples does not interfere with the PhiCal Test.

Oral Medications
No interference from relevant oral medications has been identified.
The following oral medications and nutritional supplements have been tested for potential interferences: Prednisone; Sulfamethoxazole, Pentasa; Prevacid; Vancomycin; Asacol; Azathioprine; Ciprofloxacin HCL; Ferrous Sulfate; Multiple vitamin; Vitamin E; Zelnorm. The concentration of the drug in the suspension was sufficient to approximate the concentration to be expected in a patient's stool based on the appropriate dosages and average stool volume per twenty four hours.

Gastrointestinal Bleeding
Bleeding of as much as 100 mL per day would increase the fecal calprotectin concentration by only 6 mg/L (15 µg/g).[23]

Analytical Sensitivity
The analytical sensitivity of PhiCal test is 6.25 ng/ml, which corresponds to 15.6 µg calprotectin/g feces at a sample dilution of 1:2500.

References

1. Fagerhol MK, et al. Calprotectin (The L1 leukocyte protein) in: Smith V.L. and Dedman J.R. (eds.): Stimulus response coupling: The role of intracellular calcium-binding proteins. CRC Press, Boca Raton 1990, p.187-210
2. Røseth AG, et al. Assessment of the neutrophil dominating protein calprotectin in feces. A methodologic study. Scand J Gastroenterol 1992;27:793-798.
3. Røseth AG, et al. Correlation between faecal excretion of Indium-111-labelled granulocytes and calprotectin, a granulocyte marker protein, in patients with inflammatory bowel disease. Scand J Gastroenterol 1999;34:50-54.
4. Tibble J, et al. A simple method for assessing intestinal inflammation in Crohn's disease. Gut 2000;47:506-513.
5. Bunn SK, et al. Fecal calprotectin: Validation as a noninvasive measure of bowel inflammation in childhood inflammatory bowel disease. J Pediatr Gastroenterol Nutr 2001;33:14-22.
6. Dale I, et al. Purification and partial characterization of a highly immunogenic human leukocyte protein, the L1 antigen. Eur J Biochem 1983;134:1-6.
7. Sohnle PG, et al. The zinc-reversible antimicrobial activity of neutrophil lysates and abscess fluid supernatants. Journal of Infectious Diseases 1991;164:137-142.
8. Brandtzaeg P, et al. Distribution of a formalin-resistant myelomonocytic antigen (L1) in human tissues. II. Normal and aberrant occurrence in various epithelia. American J of Clin Pathology 1987;87:700-707.
9. Fagerhol MK. Nomenclature for proteins: is calprotectin a proper name for the elusive myelomonocytic protein? J Clin Pathol: Mol Pathol 1996;49:M74-M79.
10. Isaksen B, Fagerhol MK. Calprotectin inhibits matrix metalloproteinases by sequestration of zinc. J Clin Pathol: Mol Pathol 2001;54:289-292.

11. Steinbakk M, et al. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. Lancet 1990;336:763-765.
12. Yui S, et al. Induction of apoptotic cell death in mouse lymphoma and human leukemia cell lines by a calcium-binding protein complex, calprotectin, derived from inflammatory peritoneal exudate cells. Journal of Leukocyte Biology 1997;58:650-658.
13. Tøn H, et al Improved assay for fecal calprotectin. Clinica Chimica Acta 2000;292:41-54.
14. Limburg PJ, et al. Fecal calprotectin levels predict colorectal inflammation among patients with chronic diarrhea referred for colonoscopy. Am J Gastroentrerol 2000;95:2831-2837.
15. Røseth AG, et al. Assessment of disease activity in ulcerative colitis by faecal calprotectin, a novel granulocyte marker protein. Digestion 1997;58:176-180.
16. Tibble JA, et al. Use of surrogate markers of inflammation and Rome criteria to distinguish organic from non-organic intestinal disease. Gastroenterology 2002;123:245-260.
17. Costa F, et al. Role of faecal calprotectin as non-invasive marker of intestinal inflammation. Dig Liver Dis 2003;35:642-647.
18. Carroccio A, et al. Diagnostic accuracy of fecal calprotectin assay in distinguishing organic causes of chronic diarrhea from Irritable Bowel Syndrome: A prospective study in adults and children. Clin Chem 2003;49:861-867.
19. Tibble JA et al. Surrogate markers of intestinal inflammation are predictive of relapse in patients with inflammatory bowel disease. Gastroenterology 2000;119:15-22.
20. Tibble JA et al. High prevalence of NSAID enteropathy as shown by a simple faecal test. Gut 1999;45:362-366.
21. Olafsdottir E, et al. Faecal calprotectin leels in infants with infantile colic, healthy infants, children with inflammatory bowel disease, children with recurrent abdominal pain and healthy children. Acta Paediatrica 2002;91:45-50.
22. Wassell J, et al. Faecal calprotectin: an new marker for Crohn's disease? Ann Clin Biochem 2004;41:230-232.
23. Kristinsson J, Roseth A, Fagerhol MK, Aadland E, Schjonsby H, Bormer OP, Raknerud N, Nygaard K. Fecal calprotectin concentration in patients with colorectal carcinoma. Dis Colon Rectum. 1998 Mar;41(3):316-21

Developed by:
Calpro AS,
Arnstein Arnebergsvei 30
1366 Lysaker, Norway
Under US patents 4,833,074; 5,455,160; and 6,225,072 B1.
PhiCal is a US registered trade mark, Reg. No. 1,932,460.

Manufactured by:
Eurospital S.p.A.
Via Flavia 122, 34147 Trieste, Italy
Phone (+39) 0408997.1
Fax (+39) 040280944
E-mail: info@eurospital.it

PhiCal Test
96 tests, code 9053
Date of revision: 03.19.2020
Code 32123 / Rev.2