BÜHLMANN fCAL®

ELISA

Calprotectin

For research use only.
Not for use in diagnostic procedures.

EK-CAL-U 96 tests
EK-CAL2-U 192 tests

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ENGLISH

INTENDED USE

The BÜHLMANN fCAL® ELISA is intended for the quantitative determination of calprotectin in human stool specimens.

For research use only. Not intended for use in diagnostic procedures.

PRINCIPLE OF THE ASSAY

The BÜHLMANN fCAL® ELISA allows the selective measurement of calprotectin in fecal extracts by sandwich ELISA. The microtiter plate of the BÜHLMANN fCAL® ELISA is coated with a monoclonal capture antibody (mAb) highly specific to the calprotectin heterodimeric and polymeric complexes. Fecal sample extracts, controls for determination of ELISA run acceptability, and calibrators are loaded onto wells of the microtiter plate. After a 30 minute incubation at room temperature and washing steps, a detection antibody (Ab) conjugated to horseradish peroxidase (HRP) detects the calprotectin molecules bound to the capture antibody on the plate. After incubation and further washing steps, the chromogenic HRP substrate, tetramethylbenzidine (TMB) is added (blue color formation) followed by a stopping reaction (change to yellow color). The absorption is measured at 450 nm. The final calprotectin concentration in µg/g stool in the samples is determined using the calibration curve generated from the measured calibrator values.

REAGENTS SUPPLIED AND PREPARATION

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) 2)] 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[3)] Human serum with preservatives	2 vials x 1 mL	2 vials x 1 mL	B-CAL-CONSET	Ready to use
Enzyme Label Anti-calprotectin Ab 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

[1)] The actual calprotectin concentration of the standards A to E are 4, 12, 40, 120 and 240 ng/mL, respectively. For extraction and subsequent sample dilution, a dilution of 1:2500 was taken into account for the assignment of calibrator A to E. For the lower range ELISA procedure the calibrator values have to be set as: 10, 30, 100, 300 and 600 µg/g calprotectin.

[2)] If you choose the extended range ELISA procedure the nominal calibrator values have to be set as: 30, 90, 300, 900 and 1800 µg/g calprotectin.

[3)] The controls contain lot specific amounts of native human calprotectin. Refer to the additional QC data sheet for actual concentrations.

STORAGE AND SHELF LIFE OF REAGENTS

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

Table 2

REAGENTS AND MATERIALS SUPPLIED SUPPLEMENTARY

Fecal extraction devices

Fecal extraction devices described in table 3 are not delivered with the kit and either of them has to be ordered with the kit.

Extraction Devices	Quantity	Code
CALEX® Cap Device	Package with 50, 200 or 500 tubes respectively, each filled with extraction buffer, 5 mL / ready to use	B-CALEX-C50 B-CALEX-C200 B-CALEX-C500
Smart-Prep	50 tubes, spatulas, and base caps	B-CAL-RD
ScheBo® Quick-Prep™	50 tubes consisting of tube, cone & dosing tip, 1.3 mL / ready to use	B-CAL-SO50

Table 3

MATERIALS REQUIRED BUT NOT PROVIDED

Extraction procedure

- 10 µL disposable inoculation loops
- 15 mL polypropylene tubes with screw caps required for standard extraction procedure; extraction devices (see above).
- Laminar flow work station
- Multi tube vortex mixer
- Precision balance (10-150 mg)
- Micro centrifuge (≥3000 x g)
- Centrifuge (≥500 x g)

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 to dilute the wash buffer
- Microtiter plate washer (see Technical Precautions) or squeeze bottle for wash buffer
- Microtiter plate rotator (see Technical Precautions)
- Blotting paper
- Microtiter plate reader for measurement of absorbance at 450 nm

PRECAUTIONS

Safety precautions

- The calibrators and controls of this test contain components of human origin. Although tested and found negative for HBV surface antigen, HCV and HIV1/2 antibodies, the reagents should be handled as if capable of transmitting infections and should be handled in accordance with Good Laboratory Practices (GLP) using appropriate precautions.
- Stop solution: The stop solution (B-STS12) contains sulfuric acid (0.25 M). The reagent is an irritant to eyes, skin and mucous membranes. Avoid contact with eyes, skin and clothes. After contact with eyes or skin, wash immediately with plenty of water.
- Reagents: Avoid contact of reagents with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with generous amounts of water; otherwise, irritation / burns can occur.
- Unused solution should be disposed of according to local state and federal regulations.

Technical precautions

Kit components

- Residues in the microtiter plate wells result from the production process. They are removed in the washing step (assay procedure step 3) and do not affect the results.
- Read carefully the instructions 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.
- Components must not be used after the expiry date printed on the labels.
- Do not mix different lots of reagents.
- Every effort should be made to ensure that no cross contamination occurs between reagents, samples or between wells.
- Allow the reagents to equilibrate to room temperature. Mix well (vortex) the reagents before use.
- Microwells cannot be re-used.

Extraction

- In order to receive reliable and quantitative results it is important to homogenize the stool sample entirely in the exctraction device. Contaminations at the top of the extrasction tube should be avoided. Insoluable (undigested) components may still be present after extraction.

ELISA procedure

- In the ELISA procedure the washing step is essential to guarantee reproducible results. A minimal incubation time of the wash buffer in the wells of at least 20 seconds must be ensured each time.
- When using an automated washer**,** BÜHLMANN strongly recommends using “plate mode” i.e. each process step (dispense/aspiration) is performed on all of the strips sequentially, before proceeding to the next process step. Thus, the minimal incubation time is guaranteed.
- The indicated no. of washing cycles is mandatory to ensure reproducible results.
- Set the plate rotator (shaker) at 450 rpm (<10 Hz). Higher rotation frequency may cause poor dilution linearity at values between 300/900 and 600/1800 µg/g. A rotational movement should be used instead of a horizontal movement.

- To ensure a complete antigen/antibody interaction, 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.
- 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, use only deionized high quality water.
- A new standard curve must be generated each time the assay is performed.
- If the initial concentration of an unknown sample reads above the concentration of the highest calibrator, calibrator E, the sample must be further diluted with incubation buffer and assayed again according to the assay procedure. The resulting total dilution factor must be taken into account for the calculation of results.

SPECIMEN COLLECTION AND STORAGE

If the extraction devices are used, less than 1 g of native stool specimen is needed for the extraction procedure.

Stool specimens should be collected into plain tubes.

Important: The specimen must be collected without any chemical or biological additions in the collection device.

Specimen transport
Stool specimens should be received by the laboratory within 3 days of collection. The specimens may be transported at room temperature (23 °C).

Specimen storage
Received stool specimens should be stored at 2-8 °C and extracted within 3 days.

Extract storage
Calprotectin in extracts obtained by the BÜHLMANN CALEX® Cap is stable at room temperature for 3 days, at 2-8 °C for 6 days and at -20 °C for 18 months.
Calprotectin in extracts obtained by manual weighing method, by BÜHLMANN Smart-Prep or by ScheBo® Quick-Prep™ is stable at 2-8 °C for 6 days or at -20 °C for 18 months.

STOOL SAMPLE EXTRACTION

Standard extraction procedure

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-weighted tube.
3. Calculate the net amount of sample, break off the inoculation loop and leave the lower part of the loop in the tube.
4. Add extraction buffer according to the formula
   x mg stool x 49 = y µL extraction buffer (e.g. 50 mg stool + 2450 µL buffer) to the tube and close the tube.
5. Homogenize the sample on a multi tube vortexer by vigorous shaking (at highest speed) for 30 minutes.
6. Transfer the homogenate into a 2 mL Eppendorf tube and centrifuge in a microcentrifuge for 5 minutes at 3000 x g.
7. Take the supernatant into a fresh, labeled tube and continue with the ELISA procedure.

Extraction procedures using fecal extraction devices:

The extraction procedure is described and illustrated in the instruction for use delivered with the respective extraction device.

1. CALEX® Cap Device (Code B-CALEX-C50 / B-CALEX-C200 / B-CALEX-C500): The extraction tubes are prefilled with extraction buffer.

Important: After extraction, centrifuge the CALEX® Cap Device for 5 minutes at 500-3000 x g and continue with the assay procedure.

2. Fecal extraction device Roche (Code 10745804322) or BÜHLMANN Smart-Prep (Code: B-CAL-RD).
3. ScheBo® Quick-Prep™ (Code B-CAL-SO50): The extraction tubes are prefilled with extraction buffer.

Important: After extraction with BÜHLMANN Smart Prep and ScheBo® Quick-Prep™, centrifuge the tubes for 5 minutes at 3000 x g. Alternatively, transfer the homogenate into a 2 mL Eppendorf tube and centrifuge it in a microcentrifuge for 5 minutes at 3000 x g. Decant the supernatant into a fresh, labeled tube and continue with the assay procedure.

WORKING RANGE

The assay can be performed according to the following procedures – lower or extended range ELISA procedure. Which procedure is to be chosen depends on the expected calprotectin concentration of the samples. For samples up to 600 µg/g choose the lower range procedure (working range 10-600 µg/g). If the samples tend to exceed 600 µg/g choose the extended range procedure (working range 30-1800 µg/g).

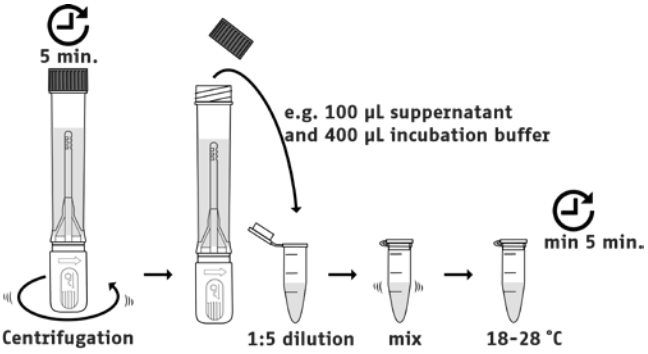
ASSAY PROCEDURE

Important: Allow the reagents to equilibrate to 18-28 °C prior to use. Only dilute stool extracts. Standards and controls are ready to use.

1. Sample dilution option 1:
Working range 10-600 µg/g

1.1. Manual weighing procedure, Smart Prep, or ScheBo® Quick-Prep™: Dilute the stool extracts 1:50 with incubation buffer (e.g. 20 µL extract and 980 µL incubation buffer) and mix well. Let the samples equilibrate for at least 5 minutes at 18-28 °C prior to proceeding to step 4c.

1.2. CALEX® Cap device: Dilute the stool extracts 1:5 with incubation buffer (e.g. 100 µL extract and 400 µL incubation buffer) and mix well. Let the samples equilibrate for at least 5 minutes at 18-28 °C prior to proceeding to step 4c.

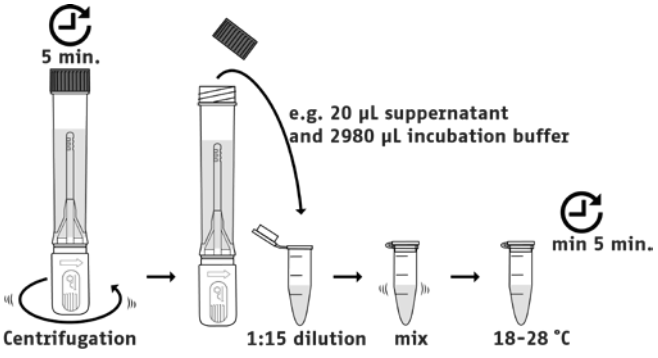


1.` Sample dilution option 2:
Working range 30-1800 µg/g

The working range can be extended by a factor of 3, if you dilute the samples 1:7500 instead of 1:2500. This procedure is recommended, if high calprotectin concentrations are to be expected. The precision, linearity and analytical sensitivity of the extended range ELISA have been validated. The results of the validation (please refer to the performance characteristics section) support the extension of the measuring range to 30-1800 µg/g.

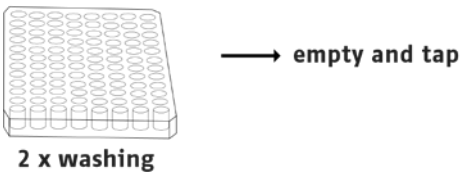
1.1` Manual weighing procedure, Smart Prep, or ScheBo® Quick Prep™: Dilute the stool extracts 1:150 with incubation buffer (e.g. 20 µL extract and 2980 µL incubation buffer) and mix well. Let the samples equilibrate for at least 5 minutes at 18-28 °C prior to proceeding to step 4c.

1.2` CALEX® Cap device: Dilute the stool extracts 1:15 with incubation buffer (e.g. 50 µL extract and 700 µL incubation buffer) and mix well. Let the samples equilibrate for at least 5 minutes at 18-28 °C prior to proceeding to step 4c.



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.
3. Wash the coated wells twice using at least 300 µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

Important: For every of the three wash steps a minimal incubation time of at least 20 seconds of the wash buffer in the wells must be ensured (see technical precautions – ELISA procedure).



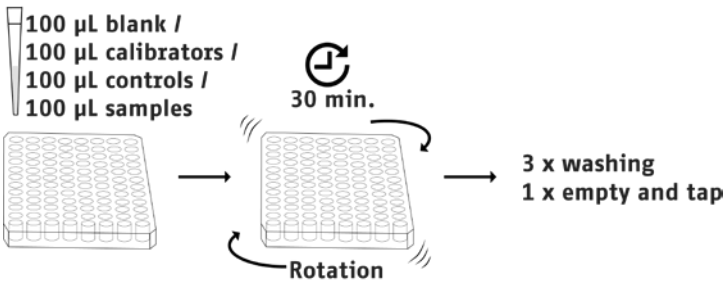
4a. Pipet 100 µL of incubation buffer (blank) and
Pipet 100 µL of Calibrator A-E into the respective wells.

4b. Pipet 100 µL of the controls low and high into the respective wells.

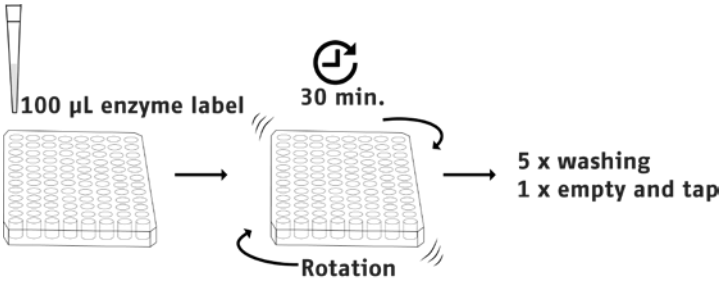
4c. Pipet 100 µL of each diluted sample into the subsequent wells.

5. Cover the plate with a plate sealer, and incubate for 30 + max. 5 min on a plate rotator set at 450 rpm at 18-28 °C (see Technical Precautions – ELISA Procedure).

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.

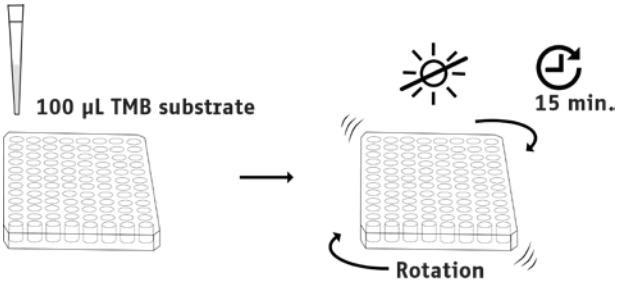


7. Pipet 100 µL of enzyme label to each well.
8. Cover the plate with a plate sealer, and incubate for 30 ±5 min on a plate rotator set at 450 rpm at 18-28 °C.
9. Remove and discard the plate sealer. Empty the wells and wash five times using at least 300 µL of wash buffer per well. Empty the wells and tap the plate firmly onto blotting paper.

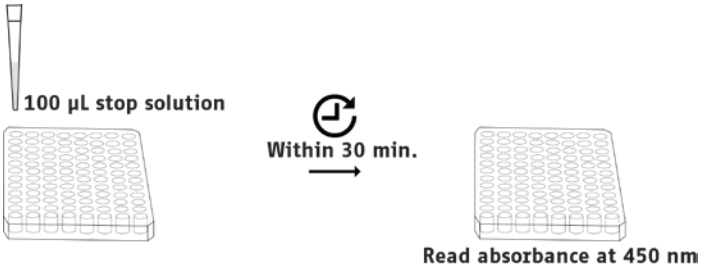


Important: Allow the TMB substrate solution to equilibrate to 18-28 °C.

10. Pipet 100 µL of the TMB substrate solution to all wells.
11. Cover the plate with a plate sealer, protect the plate from direct light and incubate for 15 ±2 min on a plate rotator set at 450 rpm at 18-28 °C.



12. Pipet 100 µL of stop solution to all wells. Remove air bubbles with a pipette tip. Proceed to step 13 within 30 min.
13. Read the absorbance at 450 nm in a microtiter plate reader.



QUALITY CONTROL

Thorough understanding of this instruction 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.
Since there is no control for calprotectin commercially available, we recommend using a pool of positive stool extracts for internal quality control.
The reproducibility of standard curve parameters and control values should be within established limits of laboratory acceptability. The confidence limits for the controls are lot-specific and printed on the additional QC data sheet.
If the performance 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 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.

STANDARDIZATION

The BÜHLMANN fCAL® ELISA calibrator values are assigned in multiple measurement runs using internal reference material based on human serum and the BÜHLMANN fCAL® ELISA measurement procedure. The calprotectin concentration of the internal reference material was established using purified MRP8/14 from human granulocytes as primary reference material.

RESULTS AND CALCULATION

Read the absorbance at 450 nm in a microplate reader for each calibrator, control and sample using a 4 PL fit with blank subtraction and have the concentration of the samples calculated.

Working range 10-600 µg/g

If you choose the lower range ELISA procedure, the calibrator concentrations have to be set as: 10, 30, 100, 300 and 600 µg/g calprotectin. Additional dilution factors (if using a different final dilution than 1:2500) have to be multiplied with the results to obtain the final results.

Refer to table 5 and figure 1 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.

Working range 30-1800 µg/g

If you choose the extended range ELISA procedure, the following nominal calibrator values have to be set as: 30, 90, 300, 900 and 1800 µg/g calprotectin. Additional dilution factors (if using a different final dilution than 1:7500) have to be multiplied with the results to obtain the final results.

Refer to table 7 and figure 5 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.

LIMITATIONS

- Reagents delivered with the BÜHLMANN fCAL® ELISA kit are intended for the determination of calprotectin levels in human stool samples only.

PERFORMANCE CHARACTERISTICS

Working range: 10-600 µg/g

Repeatability: 1.8-5.9 % CV

Within-laboratory precision: 5.3 %-10.0 % CV

Repeatability and within-laboratory precision was determined according to the CLSI approved guideline EP5-A2. Nine extracted stool samples were tested in duplicate according to assay procedure in a period of over 20 days. One run per day was performed. The values are presented in table 6.

Limit of Blank (LoB): <10 µg/g

The Limit of Blank was determined according to CLSI approved guideline EP17-A. 20 duplicates of incubation buffer were assayed in a single run with two different reagent lots. The OD values were far below that of the lowest calibrator A (10 µg/g). A cubic spline function was used to extrapolate OD values to calprotectin concentrations in µg/g.

Limit of Detection (LoD): <10 µg/g

The Limit of Detection was determined according to CLSI approved guideline EP17-A. Four stool extracts diluted to concentrations below 10 µg/g were tested in 20 replicates in two independent runs with two different reagent lots.

Functional sensitivity: <10 µg/g

Ten stool samples with values between 5.2 and 1254 µg/g calprotectin were assayed 20 times in duplicates in one assay. The % CV and the mean values were calculated for each sample. The functional sensitivity was observed at 15 % CV. The resulting precision profile (figure 2) confirms that precise measurements can be performed within the whole standard range from 10 to 600 µg/g. The results are presented in figure 2.

Linearity: 10-600 µg/g

Assay linearity was assessed according to CLSI-approved guideline EP6-A. Four extracted stool samples with concentrations higher than 600 µg/g were diluted in incubation buffer. For each sample up to 17 dilutions in the range of 1:2500 (lowest recommended dilution) up to 1:500000 were performed. Linearity results for one of the extracted stool samples are presented in figure 3.

Spiking recovery. Total bias: -1.1 %; Lower Limit of Agreement: -17.5 %, Upper Limit of Agreement: 15.4 %

Four negative extracted stool samples were spiked with increasing amounts of calprotectin from serum specimens. The results are presented in figure 4.

Working range: 30-1800 µg/g

Intra-assay precision: 2.6 %-10.5 % CV

The intra-assay precision was validated by testing twelve extracted stool samples in 20 duplicates within a single run according to the assay procedure. The values are presented in table 8.

Inter-assay precision: 7.8-12.8 % CV

The inter-assay precision of the ELISA was calculated from five extracted stool samples. The aliquots were tested according to the assay procedure in ten different runs by three technicians using two kit lots in two different labs. The values are presented in table 9.

Limit of Blank (LoB): <30 µg/g

Functional sensitivity: <30 µg/g

The intra-assay precision values obtained for 18 extracted stool samples with values between 10.8 and 2080 µg/g calprotectin were plotted against calprotectin concentration values. The resulting precision profile confirms that precise measurements can be performed within the whole standard range from 30 to 1800 µg/g. The results are presented in figure 6.

Linearity: 30-1800 µg/g

The assay linearity described for the 10-600 µg/g working range is valid for the 30-1800 µg/g working range. The 30-1800 µg/g working range is achieved by a higher sample dilution (1:7500) and different assignment of nominal calibrator values. Linearity was demonstrated using sample dilutions in the range of 1:2500-1:500000.

Spiking recovery: Total bias: -1.6 %; Lower Limit of Agreement: -11.6 %, Upper Limit of Agreement: 8.5 %.

Two negative extracted stool samples were spiked with increasing amounts of calprotectin from serum specimens. The results are presented in figure 7.

General performance characteristics

High dose hook effect

An extracted stool sample with a calprotectin concentration of 14000 µg/g and a serum sample with a calprotectin concentration of 60000 µg/g, that is 100 times higher than the highest calibrator E (600 µg/g), were tested undiluted and in serial dilutions. No high dose hook effect was observed. The results for the extracted stool sample are presented in figure 8.

Crossreactivity: <0.1 %

Incubation buffer spiked with different amounts of recombinant MRP8 and MRP14 were measured according to the assay procedure. The values are presented in table 10.

Interfering substances

The susceptibility of the BÜHLMANN fCAL® ELISA to interfering substances such as oral pharmaceuticals, nutritional supplements, hemoglobin and enteropathological microorganisms was assessed according to the CLSI-approved guideline EP7-A2. No interaction with the assay was observed. The values are presented in table 11 and table 12.

APPENDIX I

TABLES AND FIGURES

LOWER RANGE PROCEDURE 10-600 µg/g

Example of results

	Conc. [µg/g]	Absorb. [OD]	Calc. Conc. [µg/g]	CV Conc [%]
Blank Avg.		**0.096**		
Cal A	10	0.073		
Cal A	10	0.066		
Cal A Avg.	**10**	**0.069**		**7.2**
Cal B	30	0.143		
Cal B	30	0.153		
Cal B Avg.	**30**	**0.148**		**4.8**
Cal C	100	0.465		
Cal C	100	0.456		
Cal C Avg.	**100**	**0.460**		**1.4**
Cal D	300	1.121		
Cal D	300	1.135		
Cal D Avg.	**300**	**1.128**		**0.9**
Cal E	600	1.658		
Cal E	600	1.671		
Cal E Avg.	**600**	**1.664**		**0.6**
Ctrl Low		0.201	41	
Ctrl Low		0.189	39	
Ctrl Low Avg.		**0.195**	**40**	**4.4**
Ctrl High		0.598	134	
Ctrl High		0.583	130	
Ctrl High Avg.		**0.590**	**132**	**1.8**

Table 5

Example of a standard curve

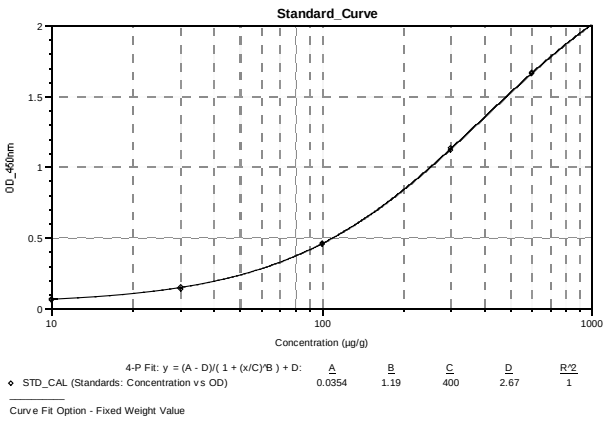


Figure 1

Within-laboratory precision

Sample No.	Concen-tration [mean µg/g]	Repeatability [%CV]	Between-day Precision [%CV]	Total Precision [%CV]
#1933	20.5	4.3	7.7	8.8
#1934	19.7	5.9	8.4	10.0
#1935	37.1	3.2	5.8	6.5
#1936	35.4	2.6	7.4	7.6
#1937	58.6	2.7	6.4	6.7
#1938	83.9	3.1	5.2	5.9
#1939	141.4	1.8	5.2	5.3
#1956	294.1	4.7	6.2	7.7
#1940	501.4	5.5	4.3	6.9

Table 6

Precision profile

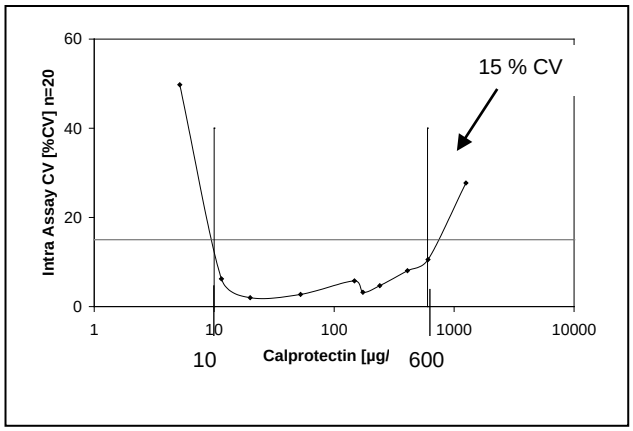


Figure 2

Linearity

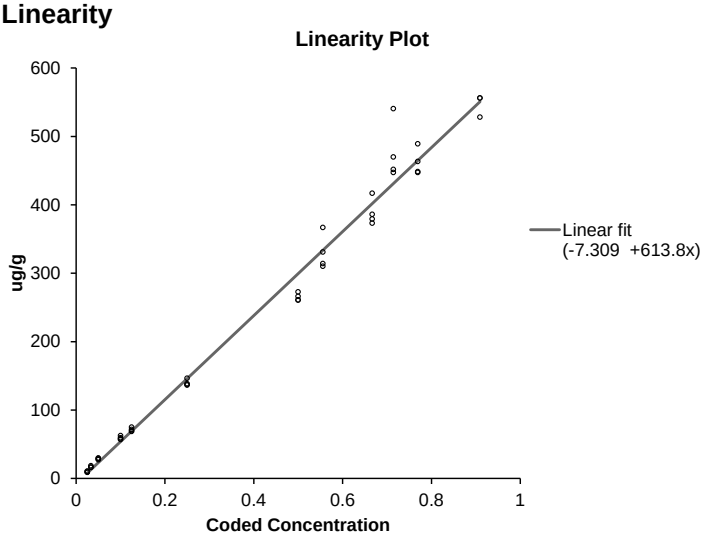


Figure 3

APPENDIX I

TABLES AND FIGURES

LOWER RANGE PROCEDURE 10-600 µg/g

Spiking recovery

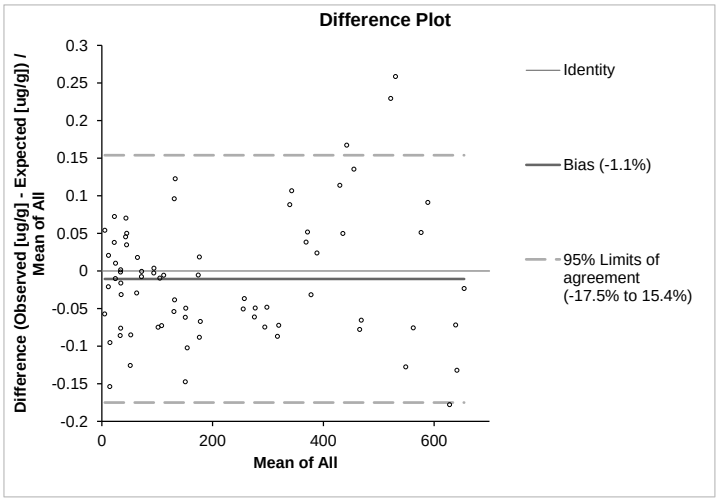


Figure 4

APPENDIX I

TABLES AND FIGURES

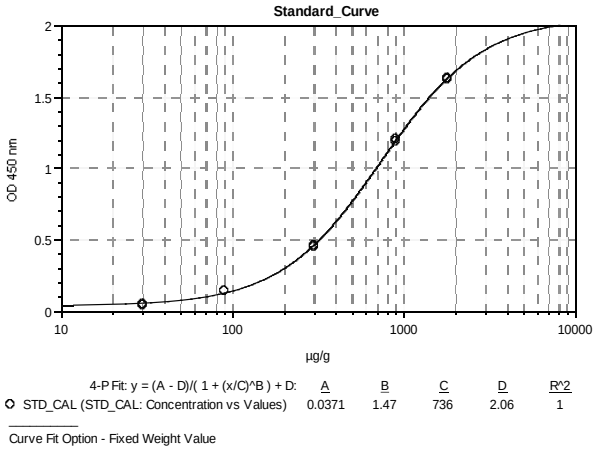
EXTENDED RANGE PROCEDURE 30-1800 µg/g

Example of results

	Conc. [µg/g]	Absorb [OD]	Calc. Conc. [µg/g]	CV Conc [%]
Blank Avg.		**0.057**		
Cal A	30	0.047		
Cal A	30	0.046		
Cal A Avg.	**30**	**0.047**		**0.9**
Cal B	90	0.138		
Cal B	90	0.140		
Cal B Avg.	**90**	**0.139**		**1.0**
Cal C	300	0.464		
Cal C	300	0.452		
Cal C Avg.	**300**	**0.458**		**1.9**
Cal D	900	1.207		
Cal D	900	1.192		
Cal D Avg.	**900**	**1.200**		**0.8**
Cal E	1800	1.627		
Cal E	1800	1.630		
Cal E Avg.	**1800**	**1.629**		**0.1**
Ctrl Low		0.147	105	
Ctrl Low		0.162	115	
Ctrl Low Avg.		**0.155**	**110**	**6.2**
Ctrl High		0.618	396	
Ctrl High		0.618	396	
Ctrl High Avg.		**0.618**	**396**	**0.6**

Table 7

Example of a standard curve



	A	B	C	D	R^2
STD_CAL (STD_CAL: Concentration vs Values)	0.0371	1.47	736	2.06	1

Figure 5

Precision, intra-assay

Specimen	n	Mean Conc. [µg/g]	SD	% CV
S6	20	46.3	4.9	10.5
S7	20	88.7	4.8	5.4
S8	20	159.1	6.2	3.9
S9	20	298.2	9.9	3.3
S10	20	412.0	11.5	2.8
S11	20	555.5	16.5	3.0
S12	20	712.9	20.1	2.8
S13	20	764.5	22.6	3.0
S14	20	1082.6	50.1	4.6
S15	20	1108.9	72.0	6.5
S16	20	1246.0	32.4	2.6
S17	20	1454.3	130.0	8.9

Table 8

Precision, inter-assay

Specimen	n	Mean Conc. [µg/g]	SD	% CV
S1	10	75.5	9.7	12.8
S2	10	224.8	19.7	8.8
S3	10	788.4	61.7	7.8
S4	10	1000.7	110.7	11.1
S5	10	1764	221.4	12.6

Table 9

Precision profile

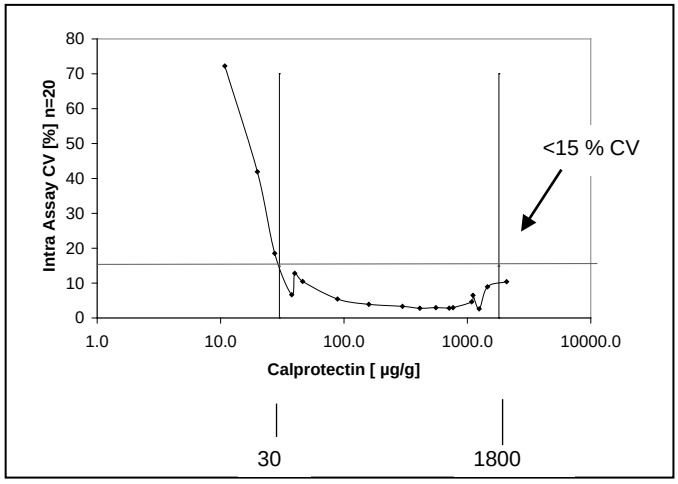


Figure 6

APPENDIX I

TABLES AND FIGURES

EXTENDED RANGE PROCEDURE: 30-1800 µg/g

Spiking recovery

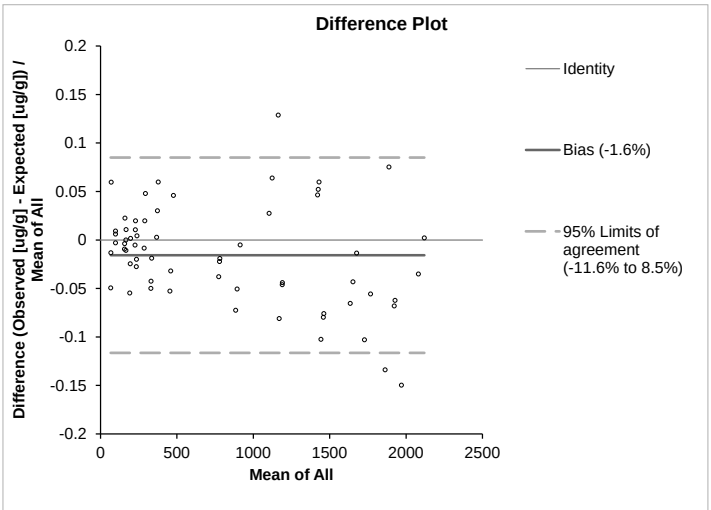


Figure 7

APPENDIX I

TABLES AND FIGURES

GENERAL PERFORMANCE CHARACTERISTICS

Cross reactivity

Spiked with	MRP8 [ng/mL]	MRP14 [ng/mL]
100 µg/mL	26.0	38.7
10 µg/mL	8.0	3.4
1 µg/mL	<4.0	<4.0
100 ng/mL	<4.0	<4.0
10 ng/mL	<4.0	<4.0

Table 10

High dose hook effect

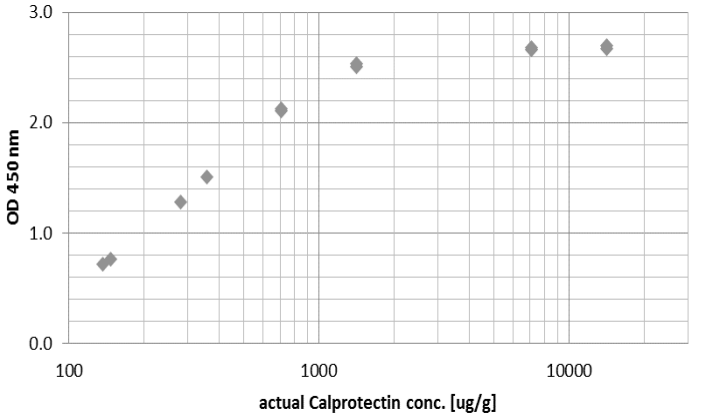


Figure 8: Stool extract with highly elevated Calprotectin concentration subsequently diluted and tested in the ELISA.

Interfering substances:
Oral pharmaceuticals, nutritional supplements and hemoglobin

Trade Name	Active component	Spiked conc.
Ferro-Gradumed	Iron (II) sulfate	0.04 mg/mL
Prednison	Prednisonum	0.13 mg/mL
Imurek	Azathioprinum	0.07 mg/mL
Pentasa	Mesalazinum; 5-ASA	2.00 mg/mL
Lansoprazol	Lansoprazolum	0.07 mg/mL
Asacol	Mesalazinum; 5-ASA	0.50 mg/mL
Zelnorm	Excluded from experiments due to market withdrawal in March 2007	
Vancomycin	Vancomycinum	0.80 mg/mL
Sulfametoxazol	Sulfametoxazol	0.64 mg/mL
Trimethoprim	Trimethoprim lactat	0.13 mg/mL
Ciprofloxacin	Ciprofloxacin	**0.08 mg/mL
Vitamin E	DL-Tocopherolacetat	0.12 mg/mL
Multiple Vitamin	A,B1,B2,B3,B5,B6,B8, B9,B12,C,D,E,+minerals	0.43 mg/mL
	Human hemoglobin	0.5 mg/mL

Table 11

*Daily maximum dose was given by the manufacturer. Spiked concentration of the compounds are based on daily volume of 150 g faeces and the possible amount in the extract resulting in a divisor of 1:7500. **Recovery of ciprofloxacin in stool was given by the manufacturer as 17.8 %.

Interfering substances: Microorganisms

Name	OD (at 600 nm) of culture.
Escherichia coli	0.87
Salmonella enterica subsp. enterica	1.81
Klebsiella pneumoniae subsp. pneumonia	1.33
Citrobacter freundii	0.64
Shigella flexneri	0.23
Yersinia enterocolitica subsp. enterocolitica	0.91

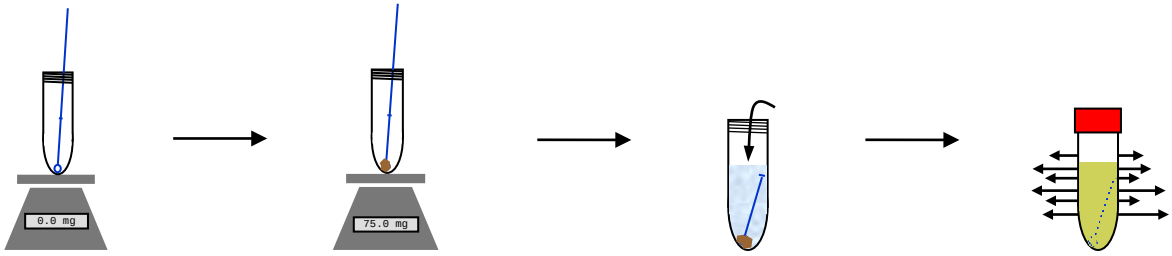
Table 12

APPENDIX III

SHORT PROTOCOL

CALPROTECTIN EXTRACTION

Standard Extraction Procedure

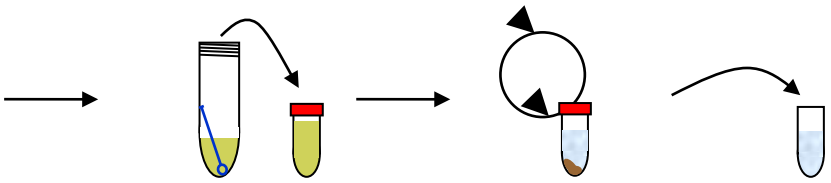


Pre-weigh empty tube + Inoculation loop

Weigh 50 to100 mg feces

Add 49 volumes of 1 x B-CAL-EX

Close tube and vortex vigorously for 30 min



Transfer ~1.0 mL into a fresh tube

Centrifuge 5 min at 3'000 x g

Transfer supernatant into a fresh tube and continue with the lower or extended range ELISA procedure (1:50 or 1:150).

APPENDIX III

SHORT PROTOCOL

CALPROTECTIN ELISA

Precoated microtiter plate

↓ ↺ *wash 2 x*

100 µL Calibrators Controls
or diluted Samples

↓ ↺ *incubate 30 (+ 5) minutes*
at 18-28 °C on a plate rotator

↓ ↺ *wash 3 x*

add 100 µL Enzyme Label

↓ ↺ *incubate 30 +/-5 minutes*
at 18-28 °C on a plate rotator

↓ ↺ ***wash 5 x***

add 100 µL TMB Substrate

↓ ↺ *incubate 15 +/-2 minutes*
at 18-28 °C on a plate rotator

add 100 µL Stop Solution

→ **Read absorbance at 450 nm (within 30 minutes)**

TIME TO RESULT: 75 MINUTES

APPENDIX V

SYMBOLS

Symbol	Explanation
	Use By
REF	Order Code
LOT	Batch code
	Contains sufficient for <n> tests
	Consult Instructions for Use-
	Temperature limitation
MP	Microtiter plate

CALEX® is a registered trademark of BÜHLMANN in many countries of the world.

Symbol	Explanation
BUF WASH 10X	Wash Buffer Concentrate (10x)
BUF INC	Incubation Buffer
CAL A - CAL E	Calibrator A -E
CONTROL L	Control Low
CONTROL H	Control High
EL	Enzyme Label
SUBS TMB	TMB Substrate
SOLN STOP	Stop Solution