Human Fecal Calprotectin

HK382

Edition 07-22

RAPID ELISA KIT
PRODUCT INFORMATION & MANUAL

Read carefully prior to starting procedures!
For use in laboratory research only
Not for clinical or diagnostic use

info@hycultbiotech.com ● www.hycultbiotech.com

Note that this user protocol is not lot-specific and is representative for the current specifications of this product. Please consult the vial label and the Certificate of Analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions.

For research use only. Not for use in or on humans or animals or for diagnostics. It is the responsibility of the user to comply with all local/state and federal rules in the use of this product. Hycult Biotech is not responsible for any patent infringements that might result from the use or derivation of this product.

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1. INTENDED USE

The fecal Calprotectin ELISA kit is to be used for the in vitro quantitative determination of human calprotectin in feces. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

Calprotectin, also known as S100A8/A9 heterocomplex, is formed out of the calcium-binding, migrating proteins S100A8 and S100A9. Calprotectin is a calcium- and zinc-binding protein complex composed of 8 and 14 kD subunits and complex formation is calcium dependent. Neutrophils are the main producers of calprotectin, but expression has also been found in monocytes, macrophages and ileal tissue eosinophils. Altogether, S100A8 and S100A9 proteins contribute to approximately 40–60% of the soluble, cytosolic content of granulocytes. Calprotectin has antibacterial, antifungal, immunomodulating and antiproliferative effects. Elevated concentrations of calprotectin can be measured among others in plasma, synovial fluid, urine and feces. The presence of calprotectin in feces quantitatively relates to neutrophil migration towards the gastrointestinal tract.

Calprotectin is therefore considered a reliable biomarker for intestinal inflammation throughout the gastrointestinal tract. Calprotectin in feces is stable for several days and even longer at 4 °C. Consistently higher fecal calprotectin levels have been reported in both adult and pediatric patients with IBD compared to IBS or healthy controls. Raised levels of the protein indicate presence of luminal white cells. Such finding indicates that the intestine is inflamed, although the type of inflammation is not defined.

The ELISA is a single incubation step reaction. After this incubation the wells are filled with substrate. The obtained color intensity is proportional to the amount of calprotectin in your sample. The amount of calprotectin can be calculated based on the standard curve of calibrated native calprotectin. The test performance is assured by the addition of a high control and a low control.

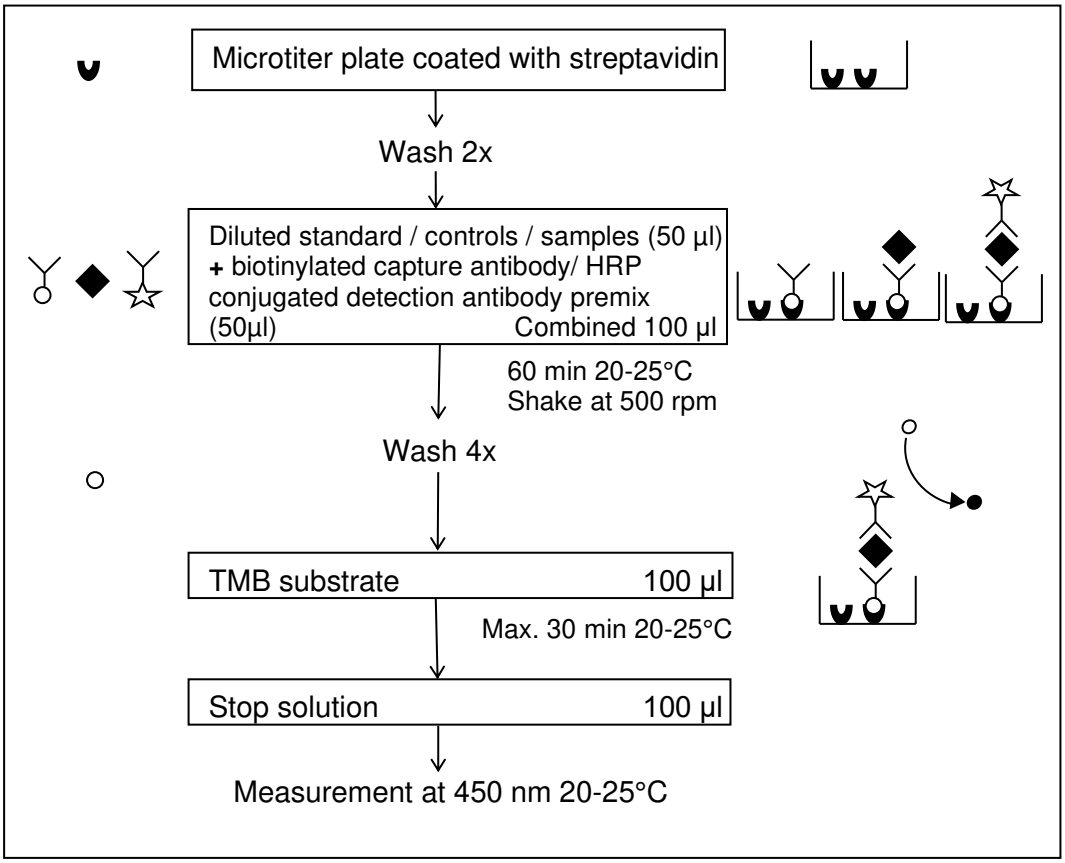
3. KIT FEATURES

- Working time of 1 hour and 30 minutes.
- Minimum concentration calprotectin which can be measured is 16 µg/g feces.
- Measurable concentration range of 16-625 µg/g (Range I; 50x sample dilution) or 48-1,875 µg/g (Range II; 150x sample dilution).
- Working volume of 100 µl/well (50 µl sample + 50 µl assay reagents).
- The assay can be performed on an automated ELISA platform which is capable to shake the plate during incubation. The assay was tested on a Dynex DS2 to evaluate test performance. Program for this platform is available on request.

Cross-reactivity

Calprotectin is a complex consisting of S100A8 and S100A9 monomers. To ensure the assay is specific, recombinant S100A8 and S100A9 were spiked in four different samples in a range of 0-10,000 ng/ml. In both cases the concentration at which an effect is measurable would be at least four times above the calprotectin level of the highest value (S1) of the standard curve (250 ng/ml). Up to 100 ng/ml S100A8 there was no effect on calprotectin measurement. At 1,000 ng/ml S100A8, obtained calprotectin level differed 103-118% compared to no spike. For S100A9 there was also no effect up to 100 ng/ml and 69-85% at 1,000 ng/ml.

4. PROTOCOL OVERVIEW



- The fecal Calprotectin ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 1 hour and 30 minutes.
- The efficient format of a plate with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Biotinylated capture antibody and HRP conjugated detection antibody will bind to calprotectin present in standard and samples in the mixture. Biotinylated capture antibody will bind to the streptavidin coated plates, in complex with the standard or samples and the HRP conjugated detection antibody.
- HRP conjugated detection antibody will react with the substrate, tetramethylbenzidine (TMB).
- The enzyme reaction is stopped by the addition of oxalic acid.
- The absorbance at 450 nm is measured with a spectrophotometer. A standard curve is obtained by plotting the absorbance (linear) versus the corresponding concentrations of the calprotectin standards (log).
- The calprotectin concentration of samples and controls, which are run concurrently with the standards, can be determined from the standard curve.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Kit component	Cat.#	Quantity HK382	Color code
Wash buffer 40x	WB01	1 vial (30 ml)	Colorless
Dilution buffer A 10x	DB77	1 vial (20 ml)	Green
Dilution buffer B 10x	DB65	1 vial (20 ml)	Blue
Standard		2 vials, lyophilized	White
Capture antibody, biotinylated 1x		1 vial, 10 ml in solution	Green
Detection antibody, HRP conjugated 300x		1 tube, 0.1 ml in solution	Brown
High control		2 vials, lyophilized	Red
Low control		2 vials, lyophilized	Blue
TMB substrate	TMB050	1 vial, (11 ml)	Brown
Stop solution	STOP110	1 vial, (11 ml)	Red
Microtiter plate, pre-coated		1 plate	
Certificate of Analysis		1	
Manual		1	
Data collection sheet		1	

Table 1

- Upon receipt, store individual components at 2 – 8 °C, do not freeze.
- Do not use components beyond the expiration date printed on the kit label.
- Lyophilized standard and controls are single use and cannot be stored after reconstitution.
- The reconstitution volume of the standard is indicated on the Certificate of Analysis.
- After opening, the capture and detection antibody are stable for one month if stored at 2 - 8°C.
- Upon receipt, foil pouch around the plate should be vacuum-sealed and unpunctured. Any irregularities to aforementioned conditions may influence plate performance in the assay.
- Return unused strips immediately to the foil pouch containing the desiccant pack and reseal along the entire edge of the zip-seal. Quality is guaranteed for one month if stored at 2 – 8 °C.
- The wash buffer and dilution buffer cannot be stored after dilution.

Materials required but not provided

- Spatula.
- Milligram weighing scale.
- Polypropylene tubes with a minimum capacity of 5 ml for extraction procedure.
- Vortex mixer.
- Centrifuge for used polypropylene tubes (>3,000 rpm).
- Calibrated micropipettes and disposable tips.
- Polypropylene tubes with a minimum capacity of 0.5 ml to 1.5 ml depending on dilution.
- Buffer preparation cilinders.
- Distilled or de-ionized water.
- Shaking device for the ELISA plate (>500 rpm).
- Microplate washer, an automated ELISA workstation is optional.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.

6. WARNINGS AND PRECAUTIONS

- This kit should only be used by qualified laboratory staff and should be familiar with ELISA procedure.
- Do not use kit components beyond the expiration date.
- Do not mix reagents from different kits and lots. The reagents have been standardized as a unit for a given lot. Use only the reagents supplied by the manufacturer unless otherwise mentioned.
- The assay has been optimized for the indicated standard range. Do not change the standard range.
- It is advised to add internal reference samples when performing the procedure.
- Open vials carefully as they can be under vacuum.
- It is advised to spin down tubes before use.
- There are no hazardous ingredients present in the product in an amount that requires labeling in accordance with EC directives or respective national laws. However, ingestion or exposure to large amounts can potentially be hazardous.
- Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse with plenty of water and soap. In all cases of doubt or when symptoms persist, always seek medical attention.
- The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse microwells or pour reagents back into their bottles once dispensed.
- Hemolyzed, hyperlipemic, heat-treated or contaminated samples may give erroneous results.
- Use polypropylene tubes for preparation of standard and samples. Do not use polystyrene tubes or sample plates.
- The standard and controls are of human origin. It was tested for various viruses and found negative. Since no test method can offer complete assurance that infectious agents are absent, this reagent should be handled as any potentially infectious human serum or blood specimen. Handle all materials in contact with this reagent according to guidelines for prevention of transmission of blood-borne infections.

7. SAMPLE PREPARATION

Collection and handling

Feces

Calprotectin can be measured in feces if samples are extracted using fecal extraction buffer. The extraction buffer can be made using the following formulation: 0.1 M Tris, 0.15 M NaCl, 1.0 M urea, 10 mM CaCl2, 0.1 M citric acid monohydrate, 5 g/l BSA and 0.25 mM thimerosal (pH 8.0). Add 4.9 ml extraction buffer to 100 mg sample (giving a dilution factor of 50, assuming the density of feces to be 1 g/ml). In case a smaller stool sample is used (min. 50 mg) the amount of extraction buffer should be adapted proportional to the amount of stool sample. Vortex samples until no large particles are present. Transfer part of the homogenate into a fresh tube and centrifuge for 5 minutes at ≥ 3,000g. When undiluted, the extract can be stored at -80 °C for at least one year. When diluted, the obtained supernatant can be used in the assay.

Storage

Store samples below -20 °C, preferably at -70 °C in polypropylene tubes before and after extraction. Storage at -20 °C can affect recovery of Calprotectin. Samples should be frozen as quickly as possible and should not be thawed until directly prior to performing the assay. Avoid multiple freeze-thaw cycles which may cause loss of Calprotectin activity and give erroneous results.

Before performing the assay, the extracted samples should be brought to room temperature (18 – 25 °C) and mixed gently. Prepare all samples (controls and test samples) prior to starting the assay procedure. Avoid foaming.

Dilution procedures

Calprotectin can be measured accurately if extracted feces samples are diluted with dilution buffer in polypropylene tubes. It is recommend to perform both a 50x and 150x dilution of extracted samples; e.g. 20 µl + 980 µl dilution buffer in case of range I and 20 µl + 2,980 µl in case of range II.

Comment regarding recommended sample dilution

The recovery of human calprotectin from an undiluted sample is not 100% and may vary from sample to sample. When testing less diluted samples it is advisable to run recovery experiments to determine the influence of the matrix on the detection of human calprotectin. Please see Table 2 for the recommended sample dilutions. Volumes are based on a total volume of at least 230 µl of diluted sample, which is sufficient for one sample in duplicate in the ELISA. For dilution of samples we recommend to use at least 10 µl of sample. Using dilutions other than the recommended 50x or 150x may lead to a different calibration range. Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

	Dilution	Pre-dilution	Amount of sample or pre-dilution required	Amount of dilution buffer required
1.	50x	Not necessary	20 µl (sample)	980 µl
2.	150x	Not necessary	20 µl (sample)	2,980 µl

Table 2

8. REAGENT PREPARATION

Allow all the reagents to equilibrate to room temperature (20 – 25 °C) prior to use. Return to proper storage conditions immediately after use.

Wash buffer

Prepare wash buffer by mixing 30 ml of wash buffer with 1,170 ml of distilled or de-ionized water, which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the wash buffer with 39 parts of distilled or de-ionized water. Mix well.

Dilution buffer: mixture of buffer A and B

Prepare dilution buffer by adding 20 ml of the 10x dilution buffer A to 160 ml distilled or de-ionized water and mix well. Finally add 20 ml of 10x dilution buffer B to the solution and mix well. This quantity is sufficient for 2 x 96 tests. In case less volume is required, prepare the desired volume of dilution buffer by separately adding 1 part of the 10x dilution buffer A and 1 part of 10x dilution buffer B to 8 parts distilled or de-ionized water. Concentrated dilution buffer may contain crystals. In case the crystals do not disappear at room temperature within 1 hour, concentrated dilution buffer can be warmed up to 37 °C. Do not shake the solution.

Standard curve

The standard is reconstituted with 0.25 ml distilled or de-ionized water. For optimal results, please allow the reconstituted standard to rest for 1 to 2 minutes. Subsequently, add the amount of dilution buffer as stated on the Certificate of Analysis in order to obtain the first standard concentration (S1). Prepare each Calprotectin standard in polypropylene tubes by dilution of the standard with dilution buffer as shown in Table 3.

Tube	Volume standard	Volume dilution buffer	Concentration Calprotectin (µg/g stool) Sample dilution:	
			Range I 50x	Range II 150x
1 (=S1)	Reconstituted vial	See Certificate of Analysis	625	1,875
2	200 µl tube 1	300 µl	250	750
3	100 µl tube 1	525 µl	100	300
4	50 µl tube 1	730 µl	40	120
5	50 µl tube 1	1,900 µl	16	48
6	Blanco	200 µl	0	0

Table 3

High control and Low control

The controls are reconstituted by pipetting 0.25 ml distilled or de-ionized water. After reconstitution 0.25 ml dilution buffer should be added.

Detection - and Capture antibody

Add 33 µl of the 300x detection antibody to 10 ml of the capture antibody which is sufficient for 1 x 96 tests. In case less volume is required, prepare the desired volume of antibody mix in a separate tube by diluting 1 part of the 300x detection antibody with 299 parts of the capture antibody.

9. ELISA PROTOCOL

Except for the sample dilution, the ELISA protocol for Range I (16-625 µg/g) is similar to Range II (48-1875 µg/g).

1. Bring all reagents to room temperature (20 – 25 °C) before use.
2. Determine the number of test wells required, put the necessary microwell strips into the supplied frame, and fill out the data collection sheet. Return the unused strips to the storage bag with desiccant, seal and store at 2 – 8 °C.
3. Prepare standard curve as described in Table 3. Make sure to let the standard rest for 1 to 2 minutes after reconstitution.
4. Prepare controls as described in chapter 8.
5. Choose in which range (range I 16-625 µg/g; range II 48-1,875 µg/g) the samples will be tested. Dilute the fecal extracts with the corresponding dilution factor in dilution buffer (resp. 50 and 150x).
   a. Range I 16-625 µg/g ⇒ 50x sample dilution; e.g. 20 µl fecal extract + 980 µl dilution buffer. Mix well
   b. Range II 48-1,875 µg/g ⇒ 150x sample dilution; e.g. 20 µl fecal extract + 2,980 µl dilution buffer. Mix well
6. When performing manually, wash the plate 2 times with wash buffer as follows:

a. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate by inverting the plate and shaking the content out over the sink, keep inverted and tap dry on a layer of tissues.
b. Repeat the washing procedure one time.

7. Transfer 50 µl in duplicate of standard, diluted samples or controls into appropriate wells. Do not touch the side or bottom of the wells.
8. Transfer 50 µl of the antibody mixture into appropriate wells. Do not touch the side or bottom of the wells.
9. Cover the tray. Tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
10. Place the plate on a shaking device with 500 rpm and incubate the strips or plate for 1 hour at room temperature.
11. When performing manually, wash the plates 4 times with wash buffer as follows:
    a. Carefully remove cover, avoid splashing.
    b. Empty the plate by inverting plate and shaking contents out over the sink, keep inverted and tap dry on a thick layer of tissues.
    c. Add 200 µl of wash buffer to each well, wait 20 seconds, empty the plate as described in 11b.
    d. Repeat the washing procedure 11b-11c three times.
    e. Empty the plate and gently tap on thick layer of tissues.
12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 7. Do not touch the side or bottom of the wells.
13. Cover the tray and incubate the tray for max. 30 minutes at room temperature. It is advised to check the reaction on the plate regularly. In case of strong development the TMB reaction can be stopped sooner. Avoid exposing the microwell strips to direct sunlight.
14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Gently tap the tray to mix the solution and to eliminate air bubbles trapped in the wells.
15. Read the plate within 30 minutes after addition of stop solution at 450 nm using a plate reader at room temperature, following the instructions provided by the instruments manufacturer.

10. INTERPRETATION OF RESULTS

- Calculate the mean absorbance for each set of duplicate standards, control and samples.
- If individual absorbance values differ by more than 15% from the corresponding mean value, the result is considered suspect and the sample should be retested.
- The mean absorbance of the zero standard should be less than 0.3.
- Create a standard curve using computer software capable of generating a good curve fit. The mean absorbance for each standard concentration is plotted on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis (logarithmic scale).
- If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
- Samples that give a mean absorbance above the absorbance for the highest standard concentration are out of range of the assay. These samples should be retested at a higher dilution.

11. TECHNICAL HINTS

- User should be trained and familiar with ELISA assays and test procedure.

- If you are not familiar with the ELISA technique it is recommended to perform a pilot assay prior to evaluation of your samples. Perform the assay with a standard curve only following the instructions.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing wash buffer, fill with wash buffer as indicated for each cycle and do not allow wells to sit uncovered or dry for extended periods.
- Since exact conditions may vary from assay to assay, a standard curve must be established for every run. Samples should be referred to the standard curve prepared on the same plate.
- Do not mix reagents from different batches, or other reagents and strips. Remainders should not be mixed with contents of freshly opened vials.
- Each time the kit is used, fresh dilutions of standard, samples, controls, detection antibody, capture antibody, solutions and buffers should be made.
- Caps and vials are not interchangeable. Caps should be replaced on the corresponding vials.
- To avoid cross-contaminations, change pipette tips between reagent additions of each standard, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Waste disposal should be performed according to your laboratory regulations.

Technical support

If you have any information or insights regarding the use or results of the products, please do not hesitate to fill out the feedback form on the website or contact our technical support team at support@hycultbiotech.com. For more information, inquiries and/or technical support you can also contact us using the following contact details:

Hycult Biotech, Frontstraat 2A, 5405 PB Uden, the Netherlands. T: +31 (0)413 251 335, F: +31 (0)413 248 353, www.hycultbiotech.com.

12. QUALITY CONTROL

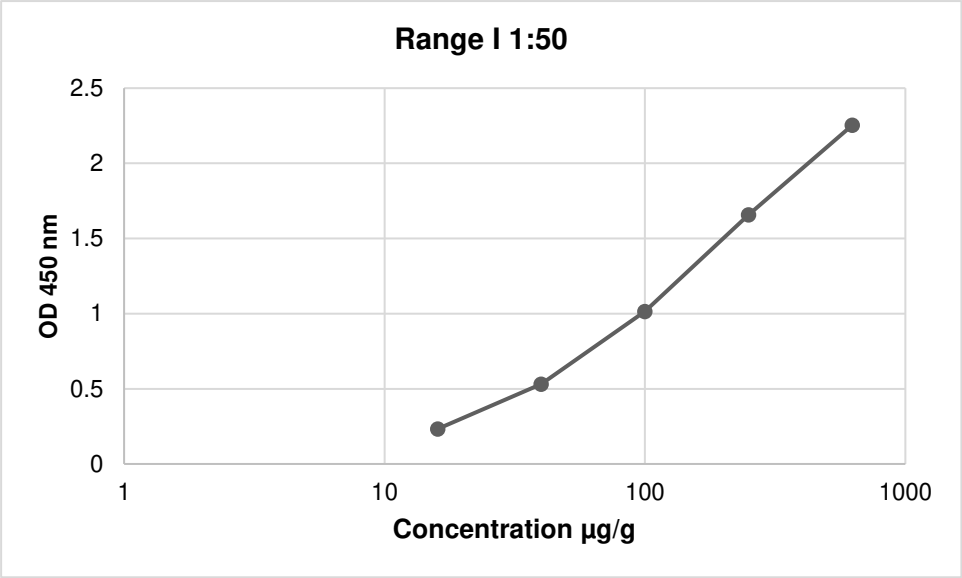
The Certificate of Analysis included in this kit is lot-specific and is to be used to verify results obtained by your laboratory. The absorption values provided on the certificate of quality control are to be used as a guideline only. The results obtained by your laboratory may differ.

This assay has been tested on specificity and interference of relevant substances. However, the possibility of interference cannot be excluded. For optimal performance of this kit, it is advised to work according to Good Laboratory Practice (GLP).

13. PERFORMANCE CHARACTERISTICS

Standard curve

Example of a typical standard curve:



Graph 1

Precision and reproducibility

The intra-assay precision and reproducibility was tested with extracts of four fecal samples using twenty identical aliquots including the high control and low control.

Intra-assay precision (n=20)	Calprotectin µg/g (min-max)	CV %
S1	67 (59-78)	3.9
S2	63 (55-71)	5.5
S3	44 (40-47)	2.8
S4	131 (110-190)	6.7
Low control µg/g	61.5	15.4
High control µg/g	240	13.1

Table 4

To determine the inter-assay variation, five samples were tested by two operators both manually and automatically (Dynex DS2) on three separate days.

Inter-assay precision (n=5)	Calprotectin µg/g (min-max)	CV %
S1	65 (56-84)	3.9
S2	73 (62-80)	5.5
S3	74 (65-86)	2.8
S4	128 (118-136)	4.5
S5	113 (97-125)	9.2

Table 5

Recovery

Recovery was determined by mixing fecal extraction samples with a known calprotectin concentration in different ratios.

Set up		Sample ratios	Observed Calprotecin [µg/g]	CV %	Expected concentration [µg/g]	Recovery %
I	S1 S2	100% 0%	40	0.3	N/A	N/A
I	S1 S2	75% 25%	45	3.7	45	100
I	S1 S2	50% 50%	52	6.6	50	96.1
I	S1 S2	25% 75%	55	2.9	51.2	93.2
I	S1 S2	0% 100%	60	2.4	N/A	N/A
II	S3 S2	100% 0%	117	3.5	N/A	N/A
II	S3 S2	75% 25%	98	8.2	102.5	104.6
II	S3 S2	50% 50%	84	6.3	88	104.8
II	S3 S2	25% 75%	71	1.6	82.5	116.2
II	S3 S2	0% 100%	59	2.1	N/A	N/A
III	S1 S3	100% 0%	38	2.0	N/A	N/A
III	S1 S3	75% 25%	56	0.2	56.5	100.9
III	S1 S3	50% 50%	73	0.6	75	102.3
III	S1 S3	25% 75%	96	3.5	81.5	84.9
III	S1 S3	0% 100%	112	3.8	N/A	N/A

Table 6

Limit of detection

The detection limit (LOD) is the lowest amount of analyte that can be detected but not necessarily quantified as an exact value. In the DH002 the LOD is formulated as the standard concentration that gives OD> OD blanc +3*(STdev OD blank). This is not necessarily equivalent to lowest standard value of the calibrator.

Limitations

An abnormal high or low concentration of fecal calprotectin does not necessarily imply the existence or absence of any disease. The values obtained from this assay are an aid to diagnosis only. Each physician must interpret these results in light of the patient's history, physical findings and other diagnostic procedures.

Special care should be taken when handling the samples to be measured. Samples should be frozen as quickly as possible and should not be thawed until directly prior to performing the assay.

14. TROUBLESHOOTING

Warranty claims and complaints in respect of deficiencies must be logged before expiry date of the product. A written complaint containing lot number of the product and the generated data can be sent to support@hycultbiotech.com.

Suggestions summarized below in Table 7 can be used as guideline in case of unexpected assay results.

Low absorbance	High absorbance	Poor duplicates	All wells positive	All wells negative	Possible cause
•	•		•	•	Kit materials or reagents are contaminated or expired
•					Incorrect reagents used
•		•	•		Lyophilized reagents are not properly reconstituted
•	•	•	•	•	Incorrect dilutions or pipetting errors
•		•			Improper plastics used for preparation of standard and/or samples
•	•				Improper incubation times or temperature
		•			Especially in case of 37°C incubation: plates are not incubated uniformly
•					Assay performed before reagents had reached room temperature
•	•	•	•	•	Procedure not followed correctly
				•	Omission of a reagent or a step
		•			Poor mixing of samples
	•		•		Low purity of water
	•	•			Strips were kept dry for too long during/after washing
	•	•	•		Inefficient washing
	•	•			Cross-contamination from other samples or positive control
		•	•		TMB solution is not clear or colorless
•	•				Wrong filter in the microplate reader
	•	•			Air bubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions
•					Lamp in microplate reader is not functioning optimally

Table 7