Human Calprotectin Stool ELISA Kit

Cat.No: DEIA2223
Lot. No. (See product label)

Size

96T

Intended use

The described Calprotectin ELISA is intended for the quantitative determination of calprotectin (MRP (8/14, S100A8/A9)) in stool. For in vitro diagnostic use only.

General Description

Calprotectin is a calcium-binding protein secreted predominantly by neutrophils and monocytes. Fecal calprotectin is a marker for neoplasic and inflammatory gastrointestinal diseases. It is often difficult to distinguish between irritable bowel syndrome and chronic inflammatory bowel disease. This leads in many cases to extensive and unnecessary colonoscopic examinations. The calprotectin test allows clear differentiation between the two patient groups. Fecal calprotectin levels correlate significantly with histologic and endoscopic assessment of disease activity in Morbus Crohn's disease and ulcerative colitis as well as with the fecal excretion of indium-111-labelled neutrophilic granulocytes that has been suggested as the "gold standard" of disease activity in inflammatory bowel disease. However, measuring 111-indium-labeled granulocytes is very costly (patient's hospitalization, analysis and disposal of isotopic material) and is connected with radioactive exposition of the patients. For this reason, a repeated application to children and pregnant women is not recommended. Elevated levels of calprotectin are a much better predictor of relapse than standard inflammatory markers (CRP, ESR HB). Comparing this marker with standard fecal occult blood screening in colorectal cancer demonstrates clearly the diagnostic advantages of the fecal calprotectin test. The parameter is of a high diagnostic value: if the calprotectin level in stool is low, the probability is high that no organic intestinal disease exists.

Principle Of The Test

The assay utilizes the two-site sandwich technique with two selected monoclonal antibodies that bind to human calprotectin. Standards, controls and diluted patient samples which are assayed for human calprotectin are added to wells of microplate coated with a high affine monoclonal anti- human calprotectin antibody. During the first incubation step, calprotectin in the samples is bound by the immobilized antibody. Then a peroxidase labeled conjugate is added to each well and the following complex is formed: capture antibody - human calprotectin - Peroxidase conjugate. Tetramethylbenzidine (TMB) is used as a substrate for peroxidase. Finally, an acidic stop solution is added to terminate the reaction. The color changes from blue to yellow. The intensity of the yellow color is directly proportional to the calprotectin concentration of sample. A dose response curve of the absorbance unit (optical density, OD) vs. concentration is generated, using the values obtained from standard. Calprotectin present in the patient samples is determined directly from this curve.

Reagents And Materials Provided

1. PLATE - Holder with precoated strips: 12 x 8 wells

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2. WASHBUF - ELISA wash buffer concentrate 10x: 2 x 100 mL
3. Extract - Extraction buffer concentrate Extract, 2.5x: 1 x 100 mL
4. SAMPLEBUF - Sample dilution buffer, ready to use: 1 x 100 mL
5. STD - Calprotectin standards, lyophilized (0; 13; 52; 210; 840 ng/mL): 2 x 5 vials
6. CTRL 1 - Control, lyophilized (see specification for range): 2 x 1 vial
7. CTRL 2 - Control, lyophilized (see specification for range): 2 x 1 vial
8. CONJ - Conjugate, ready to use: 15 mL
9. SUB - TMB substrate (Tetramethylbenzidine), ready to use: 15 mL
10. STOP - ELISA stop solution, ready to use: 15 mL

Materials Required But Not Supplied

1. Ultra pure water*
2. Laboratory balance
3. Calibrated precision pipettors and 10-1000 µL tips
4. Foil to cover the microtiter plate
5. Multi-channel pipets or repeater pipets
6. Centrifuge, 3000 g
7. Vortex
8. Standard laboratory glass or plastic vials, cups, etc.
9. Microtiter plate reader

* CD AG recommends the use of Ultra Pure Water (Water Type 1; ISO 3696), which is free of undissolved and colloidal ions and organic molecules (free of particles > 0.2 m) with an electrical conductivity of 0.055 S/cm at 25°C (≤ 18.2 M cm).

Specimen Collection And Preparation

Raw stool

Calprotectin in stool is described to be stable for at least 3 days at room temperature. Nevertheless, we recommend storing the samples for no more than 48 h at 2-8°C. Long term storage is recommended at -20°C. Allow frozen samples to thaw slowly, preferably at 2-8°C, and warm the samples to room temperature before analysis. Avoid repeated freezing and thawing of the sample. Freezing can cause neutrophil granulozytes in the stool sample to burst and release calprotectin. Therefore frozen samples can be expected to contain slightly elevated concentrations of calprotectin compared to fresh samples.
Chemical or biological additives in stool sample tubes may interfere with Calprotectin. Therefore use only empty tubes or tubes filled with the extraction buffer Extract supplied by CD.

Stool extracts
Stool extract is stable for nine days at room temperature, 2-8°C or -20°C. Avoid more than three freeze-thaw cycles.

Extraction of the stool sample
Diluted extraction buffer Extract is used as a sample extraction buffer. We recommend the following sample preparation:

Stool Sample Application System (SAS)
Stool sample tube - Instructions for use
Please note that the dilution factor of the final stool suspension depends on the amount of stool sample used and the volume of the buffer.

SAS with 1.5 mL buffer:

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Applied amount of stool: 15 mg
Buffer Volume: 1.5 mL
Dilution Factor: 1:100

Please follow the instructions for the preparation of stool samples using the SAS as follows:
a) The raw stool sample has to be thawed. For particularly heterogeneous samples we recommend a mechanical homogenisation using an applicator, inoculation loop or similar device.
b) Fill the **empty sample tube** with **1.5 mL** of ready-to-use Extract extraction buffer before using it with the sample. Important: Allow the extraction buffer to reach room temperature.
c) Unscrew the tube (yellow part of cap) to open. Insert yellow dipstick into sample. The lower part of the dipstick has notches which need to be covered completely with stool after inserting it into the sample. Place dipstick back into the tube. When putting the stick back into the tube, excess material will be stripped off and leave 15 mg of sample to be diluted. Screw tightly to close the tube.
d) Shake the tube well until no stool sample remains in the notches. Important: Please make sure that you have a maximally homogenous suspension after shaking. Especially with more solid samples, soaking the sample in the tube with buffer for app. 10 minutes improves the result.
e) Allow sample to stand for app. 10 minutes until sediment has settled. Floating material like shells of grains can be neglected.
f) Carefully unscrew the complete cap of the tube including the turquoise ring plus the dipstick. Discard cap and dipstick. Make sure that the sediment will not be dispersed again.

Dilution I: 1:100
Dilution of samples
The suspension of the sample preparation procedure (dilution I) is diluted **1:25 with SAMPLEBUF** (sample dilution buffer). For example:

40 µL suspension (dilution I) + **960 µL** SAMPLEBUF = **1:25 (dilution II)**
This results in a final dilution of 1:2500.
For analysis, pipet **100 µL** of **dilution II** per well.

Reagent Preparation

To run assay more than once, ensure that reagents are stored at conditions stated on the label. **Prepare only the appropriate amount necessary for each run**. The kit can be used up to 4 times within the expiry date stated on the label.

Reagents with a volume less than **100 µL** should be centrifuged before use to avoid loss of volume.

Preparation of the wash buffer: The wash buffer concentrate (WASHBUF) should be diluted with ultra pure water **1:10** before use (100 mL WASHBUF + 900 mL ultra pure water), mix well. Crystals could occur due to high salt con- centration in the stock solutions. The crystals must be redissolved at room temperature or in a water bath at 37°C before dilution of the buffer solutions. The **WASHBUF** is stable at 2-8°C until the expiry date stated on the label. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed flask at **2-8°C for one month**.

Preparation of the extraction buffer: The **extraction buffer concentrate Extract** must be diluted with ultra pure water **1:2.5** before use (100 mL Extract + 150 mL ultra pure water), mix well. Crystals could occur due to high salt concentration in the stock solutions. Before dilution, the crystals must be redissolved at 37°C in a water bath. The **Extract** is stable at 2-8°C until the expiry date stated on the label. Extraction buffer (1:2.5 diluted Extract) can be stored in a closed flask at **2-8°C for three months**.

The lyophilized **STD** (standards) and **CTRL** (controls) are stable at **2-8°C** until the expiry date stated on the label. The **STD** (standards) and **CTRL** (controls) must be reconstituted with **500 µL of ultra pure water**. Allow the vial content to dissolve for 10 minutes at room temperature, and mix thoroughly by gentle inversion to insure complete reconstitution. **Reconstituted standards**

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and controls can be stored at 2-8°C for 4 weeks.

All other test reagents are ready to use. Test reagents are stable until the expiry date (see label of test package) when stored at **2-8°C**.

Assay Procedure

For automated ELISA processors the given protocol may need to be adjusted according to the specific features of the respective automated platform. For further details please contact your supplier or CD AG.
We recommend to carry out the tests in duplicate.

1. Bring all reagents and samples to room temperature (15-30°C) and mix well.
2. Mark the **positions of STD/SAMPLE/CTRL** (standard/samples/controls) on a protocol sheet.
3. Take as many **microtiter strips** as needed from kit. Store unused strips covered at 2-8°C. Strips are stable until expiry date stated on the label.
4. Add **100 µL of STD/SAMPLE/CTRL** into respective well.
5. Cover plate tightly and **incubate for 30 minutes at room temperature (15-30°C)**.
6. Aspirate the contents of each well. Wash each well **5 x** with **250 µL of wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
7. Add **100 µL CONJ** (conjugate) into each well.
8. Cover plate tightly and incubate for **30 minutes at room temperature** (15-30°C).
9. Aspirate the contents of each well. Wash each well **5 x** with **250 µL of wash buffer**. After the final washing step, the inverted microtiter plate should be firmly tapped on absorbent paper.
10. Add **100 µL of SUB** (substrate) into each well.
11. Incubate for **10-20 minutes at room temperature** (15-30°C) in the dark*.
12. Add **100 µL of STOP** (stop solution) into each well, mix thoroughly.
13. Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm (or 690 nm) as a reference. If no reference wavelength is available, read only at 450 nm. If the extinction of the highest standard exceeds the range of the photometer, absorption must be measured immediately at **405 nm** against 620 nm as a reference.

* The intensity of the color change is temperature sensitive. We recommend to observe the color change and to stop the reaction upon good differentiation.

Quality Control

CD recommends the use of external controls for internal quality control, if possible.
Control samples should be analysed with each run. Results, generated from the analysis of control samples, should be evaluated for acceptability using appropriate statistical methods. The results for the patient samples may not be valid if within the same assay one or more values of the quality control sample are outside the acceptable limits.

Interpretation Of Results

The following algorithms can be used alternatively to calculate the results. We recommend using the 4 parameter algorithm.

1. **4 parameter algorithm**
It is recommended to use a linear ordinate for the optical density and a logarithmic abscissa for the concentration. When using a logarithmic abscissa, the zero calibrator must be specified with a value less than 1 (e. g. 0.001).

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2. **Point-to-point calculation**
We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

3. **Spline algorithm**
We recommend a linear ordinate for the optical density and a linear abscissa for the concentration.

The plausibility of the pairs of values should be examined before the automatic evaluation of the results. If this option is not available with the used program, a control of the paired values should be done manually.

Stool samples
The obtained calprotectin levels of the stool samples have to be multplied with the **dilution factor** of **2500** (dilution I × dilution II). In case **another dilution factor** has been used, multiply the obtained result with the dilution factor used.

Reference Values

1 g stool is equivalent to 1 mL.
The median value in healthy adults is about 25 mg/kg.
Samples with a calprotectin concentration < 50 mg/kg are regarded as negative.
Samples with a calprotectin concentration between 50 mg/kg and 100 mg/kg are regarded as borderline positive. We recommend repeating the measurement at a later time point in order to confirm the result.
Samples with a calprotectin concentration > 100 mg/kg are regarded as positive.

We recommend each laboratory to establish its own reference concentration range.
Note: Many confounding factors can cause increased levels of fecal calprotectin in the absence of IBD or IBD in a quiescent disease phase, e. g. use of NSAIDs (non steroidal anti inflammatory drugs), any intercurrent gastrointestinal infection, and the presence of malignancies. These factors should be considered in the interpretation of the test results and therapy of IBD.

Precision

Intra-Assay (n = 20) CV: 3.2-5.6%
Inter-Assay (n = 12) CV: 4.4-8.9%

Sensitivity

Limit of blank, LoB: 0.957 ng/mL
Limit of detection, LoD: 1.023 ng/mL
Limit of quantitation, LoQ: 7.161 ng/mL

The evaluation was performed according to the CLSI guideline EP-17-A2. The specified accuracy goal for the LoQ was 20 % CV.

Specificity

No cross reactivity was observed to the following plasma proteins:
- Lysozyme 0%
- PMN-Elastase 0%
- Myeloperoxidase 0%
- Lactoferrin 0%

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Recovery

Dilution recovery

Three patient samples were diluted with SAMPLEBUF and analyzed. The results are shown below (n = 3):

Sample	Dilution	Calprotectin expected [mg/l]	Calprotectin measured [mg/l]
A	1:2500		820
	1:5000	410	425
	1:10000	205	192
B	1:2500		1120
	1:5000	560	561
	1:10000	280	266.5
C	1:1250		643.0
	1:2500	321.5	344.0
	1:5000	160.8	157.8
	1:10000	80.4	80.0
	1:20000	40.2	39.7
	1:40000	20.1	17.6
	1:80000	10.0	9.5
	1:160000	5.0	5.0

Spiking Recovery

Two samples were spiked with three different calprotectin concentrations and measured using this assay. (n = 2):

Sample	Unspiked Sample [µg/ml]	Spike [µg/ml]	Calprotectin expected [ng/ml]	Calprotectin measured [ng/ml]
1	18	10.5	28.5	29.6
	18	17.5	35.5	35.6
	18	40.5	58.5	59.3
	18	63.3	81.3	83.2
	18	173.2	191.2	188.7
2	20.7	10.5	31.2	33.3
	20.7	17.5	38.2	41.0
	20.7	40.5	61.2	62.7
	20.7	63.3	84.0	90.8

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Precautions

1. All reagents in the kit package are for in vitro diagnostic use only.
2. Control samples should be analyzed with each run.
3. Human materials used in kit components were tested and found to be negative for HIV, Hepatitis B and Hepatitis C. However, for safety reasons, all kit components should be treated as potentially infectious.
4. Kit reagents contain sodium azide or Proclin as bactericides. Sodium azide and Proclin are toxic. Substrates for the enzymatic color reactions are toxic and carcinogenic. Avoid contact with skin or mucous membranes.
5. The stop solution consists of diluted sulphuric acid, a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves, eye protection, and appropriate protective clothing. Any spill should be wiped out immediately with copious quantities of water. Do not breath vapour and avoid inhalation.

TECHNICAL HINTS

1. Do not interchange different lot numbers of any kit component within the same assay. Furthermore we recommend not to assemble wells of different microtiter plates for analysis, even if they are of the same batch as wells from already opened microtiter plates are exposed to different conditions as sealed ones.
2. Reagents should not be used beyond the expiration date stated on kit label.
3. Substrate solution should remain colourless until use.
4. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
5. Avoid foaming when mixing reagents.
6. The assay should always be performed according the enclosed manual.

Limitations

Samples with concentrations above the measurement range (see definition below) must be further diluted and re-assayed. Please consider this greater dilution when calculating the results.
Samples with concentrations lower than the measurement range (see definition below) cannot be clearly quantified.
The upper limit of the measurement range can be calculated as:
highest concentration of the standard curve × sample dilution factor to be used
The lower limit of the measurement range can be calculated as:
LoB × sample dilution factor to be used

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