

# CALPRO Calprotectin ELISA Test (ALP)



## 1. INTENDED USE

The **CALPRO Calprotectin ELISA Test (ALP)** is a quantitative method for the determination of calprotectin in stool samples and can be used as an aid in identifying organic disease of the small intestine, large bowel or the stomach in patients, to determine the disease activity and monitor the response to treatment in patients with ulcerative colitis or Crohn's disease. In literature Calprotectin determination has also been done in other body fluids, secretions and excretions, for instance serum, plasma or urine. The calprotectin concentrations and protocols vary and have to be performed regarding the publications (Johns et al., 1997). The CALPRO Calprotectin ELISA Test (ALP) has only been validated for stool samples.

In patients under treatment a normal calprotectin value is an indicator that mucosal healing has been achieved. The test can also be used to predict clinical relapses. Functional disorders like irritable bowel disease, do not give increased faecal calprotectin concentrations.

**The test is for *In vitro* Diagnostic use.**

## 2. BACKGROUND

Various types of organic diseases in the gastrointestinal tract may cause damage to the intestinal epithelial 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 microorganisms releasing substances which may be toxic or chemotactic, i.e. they stimulate leukocytes, in particular polymorphonuclear neutrophil granulocytes (PMN), 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 (Fagerhol et al., 1990) and can reliably be estimated in faecal samples stored for seven days at ambient temperature (Røseth et al. 1992).

Calprotectin is a 36 kilodalton calcium and zinc binding protein (Dale et al., 1983), produced by PMNs, monocytes and squamous epithelial cells except those in normal skin (Dale et al., 1985, Brandtzaeg et al., 1987). After binding of calcium it can resist degradation by leukocytic and microbial enzymes (Røseth et al., 1992, Fagerhol, 1996). By competing with different enzymes for limited, local amounts of zinc, calprotectin can inhibit many zinc dependent enzymes (Isaksen and Fagerhol, 2001) and thereby kill microorganisms or animal and human cells in culture (Steinbakk et al. 1991, Yui et al., 1995). Different types of disease, for instance bacterial infections, rheumatoid arthritis or cancer lead to activation of PMNs and increased levels of calprotectin in plasma, cerebrospinal fluid, synovial fluid, urine or other human materials (Johns et al., 1997).

It is of special importance that the concentration of calprotectin in faeces is correlated with the number of PMNs migrating into the gut lumen, and that it can be detected reliably even in small (less than one gram) random stool samples (Røseth et al., 1992 Tøn et al., 2000). Furthermore, organic diseases of the bowel give a strong calprotectin signal, i.e. elevations are regularly five to several thousand times the upper reference in healthy individuals (Røseth et al., 1992, Tibble et al., 2000, Bunn et al., 2001, Bjarnason and Sherwood, 2001) 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. Since further diagnostic procedures may be complex, expensive or expose the patient to pain, ionizing radiation or other risks, there is a need for a simple, non-invasive, inexpensive and objective method which can help in selecting patients for additional examination, for instance endoscopy. The latter normally requires general anaesthesia in children. Many studies have shown that a test for faecal calprotectin can serve this purpose. Since abdominal symptoms are very common both in children and adults, a negative **CALPRO Calprotectin ELISA Test** can save many endoscopies and thereby also money.

Inflammatory bowel diseases (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. The **CALPRO Calprotectin ELISA Test** can with high probability rule out non-inflammatory bowel disorders (Tibble et al., 2000) on the one side, and contribute to an earlier diagnosis of IBD on the other side since the test is regularly positive in active IBD. The concentration of calprotectin in stools is a non-invasive and objective marker that can be used to determine the disease activity and response to treatment of IBD and to tell when a true remission has been achieved. Many patients in clinical and with normal clinical indices for IBD have increased faecal calprotectin which is associated with low degree inflammation and increased risk of clinical relapse. Also, this inflammation is known to cause bowel strictures which may require repeated resections.

### 3. PRINCIPLE OF THE TEST

The **CALPRO Calprotectin ELISA Test** is based upon preparation of an extract of about 0.1 gram faeces mixed with about 5 ml of extraction buffer in a closed tube. After centrifugation, a sample from the supernatant is tested by an enzyme immunoassay specific for calprotectin.

The immunoassay implies that samples and standards are incubated in separate microtiter wells coated with polyclonal antibodies against calprotectin. After incubation and washing of the wells, bound calprotectin is allowed to react with immunoaffinity purified enzyme labelled anti-calprotectin antibodies. Thus the amount of enzyme bound is roughly proportional to the amount of calprotectin in the sample or standard, which can be determined by incubation with a substrate for the enzyme.

The rabbit antibodies used in the **CALPRO Calprotectin ELISA Test** react with at least six different epitopes on calprotectin which will ensure a positive signal even if some epitopes are damaged or hidden due to complex formation with other substances in the stool. The **CALPRO Calprotectin ELISA Test** is run on stool extracts prepared by the use of a patented extraction buffer which brings calprotectin into solution in a molecular configuration like that in leukocyte extracts or plasma. This is important because quantitative immunoassays require that proteins in the standards and samples have the same configuration. The use of simple extraction buffers gives calprotectin mainly in very high molecular complexes which violates this requirement; such buffers also give much lower yields during extraction of samples.

### 4. MATERIALS

#### 4.1. Reagents supplied with the kit

- **Calprotectin-Antibody coated Microassay Plate**, 12 strips, 8 wells per strip, coated with polyclonal rabbit antibodies specific for calprotectin. The plate is stored in a sealed bag with desiccant.
- **Sample diluent solution (10x conc.)\*\***, 20 ml 10X concentrate to be diluted with distilled water, pH  $8.0 \pm 0.2$  yellow colored with a blue cap.
- **Washing solution (20x conc.)\***, 2 x 50 ml 20X concentrate, to be diluted with distilled water for washing the wells; (pH  $8.0 \pm 0.2$ ) white cap.
- **Extraction solution (2,5x conc.)**, 2 x 90 ml 2.5X concentrate, to be diluted with distilled water pH  $8.0 \pm 0.2$  with a white cap
- **Enzyme conjugated antibody**, 6 ml alkaline phosphatase labelled, immunoaffinity purified IgG antibodies (from rabbit) against calprotectin in a buffer solution with sodium azide as a preservative, red colored with a black cap.
- **pNPP Enzyme substrate solution**, 12 ml, ready for use with a yellow cap.
- **Calprotectin Standards\*\***, 8 vials with 0.6 ml ready to use solution with a red cap:

Standard A:	7.8	ng/ml
Standard B:	15.6	ng/ml
Standard C:	31.3	ng/ml
Standard D:	62.5	ng/ml
Standard E:	125	ng/ml
Standard F:	250	ng/ml
Standard G:	500	ng/ml
Standard H:	1000	ng/ml
- **Calprotectin Control\*\***, ready for use, 0.6 ml with green cap.

\* contains 0.01 % Kathon after dilution

\*\* contains 0.1 % Kathon

#### 4.2. Materials supplied

- 2 Cover foils
- 1 Strip holder
- 1 Test protocol
- 1 distribution and identification plan

### 4.3. Materials required but not supplied

- Faeces collection tubes and transport container.
- Disposable, breakable inoculation loops.
- Eppendorf tubes, 1.0 to 1.5 ml.
- Sensitive digital scale (40 to 150 mg).
- Shaker.
- Microcentrifuge (10.000g)
- Freezer (-20 °C)
- Repetitive Pipettor, 50 – 200 µl.
- Timer
- Microplate reader, filter 405 nm.
- Microplate well washer.
- Pipettes to deliver volumes between 10 and 1000 µl
- Vortex mixer.
- Disposable polystyrene screw cap tubes, 14 ml
- Distilled water
- 1 M NaOH

### 5. STABILITY AND STORAGE

The reagents are stable up to the expiry date stated on the label when stored at 2 - 8 °C.

Working solution of washing buffer, sample dilution buffer and extraction buffer can be stored at 2 - 8°C for three months. Avoid exposure to high temperature and direct sunlight.

### 6. REAGENT PREPARATION

It is very important to bring all reagents, samples and controls to room temperature (18 - 25°C) before starting the test run!

#### 6.1. Coated snap-off strips

The ready to use breakapart snap-off strips are coated polyclonal rabbit antibodies specific for calprotectin. Immediately after removal of strips, the remaining strips should be resealed in the aluminium foil along with the desiccant supplied and stored at 2 - 8 °C.

#### 6.2. Enzyme conjugated antibody

The bottle contains 6 ml of a solution of alkaline phosphatase labelled, immunoaffinity purified IgG antibodies (from rabbit) against calprotectin, buffer, stabilizers, preservatives and an inert red dye. The solution is ready to use.

#### 6.3. Standards and Controls

The vials labelled with Standard A – H and controls contain 0.6 ml each of a ready to use solution.

#### 6.4. Extraction solution

Dilute the concentrated extraction buffer by adding 1 part (90 ml) to 1.5 parts (135) ml distilled water to obtain 225 ml working solution. Mix well. Store the diluted extraction buffer in a closed vessel.

#### 6.5. Washing Solution

Dilute concentrated washing buffer by adding 1 part (50 ml) to 19 parts (950 ml) distilled water to a final volume of 1000 ml.

#### 6.6. Sample diluent solution

Dilute concentrated sample diluent solution by adding 1 part (20 ml) to 9 parts (180 ml) distilled water to a final volume of 200 ml. Store the diluted extraction buffer in a closed vessel.

#### 6.7. pNPP substrate solution

The bottle contains 12 ml of *p*-nitrophenylphosphate (pNPP). The solution is ready to use and has to be kept away from the light

### 7. SPECIMEN COLLECTION AND PREPARATION

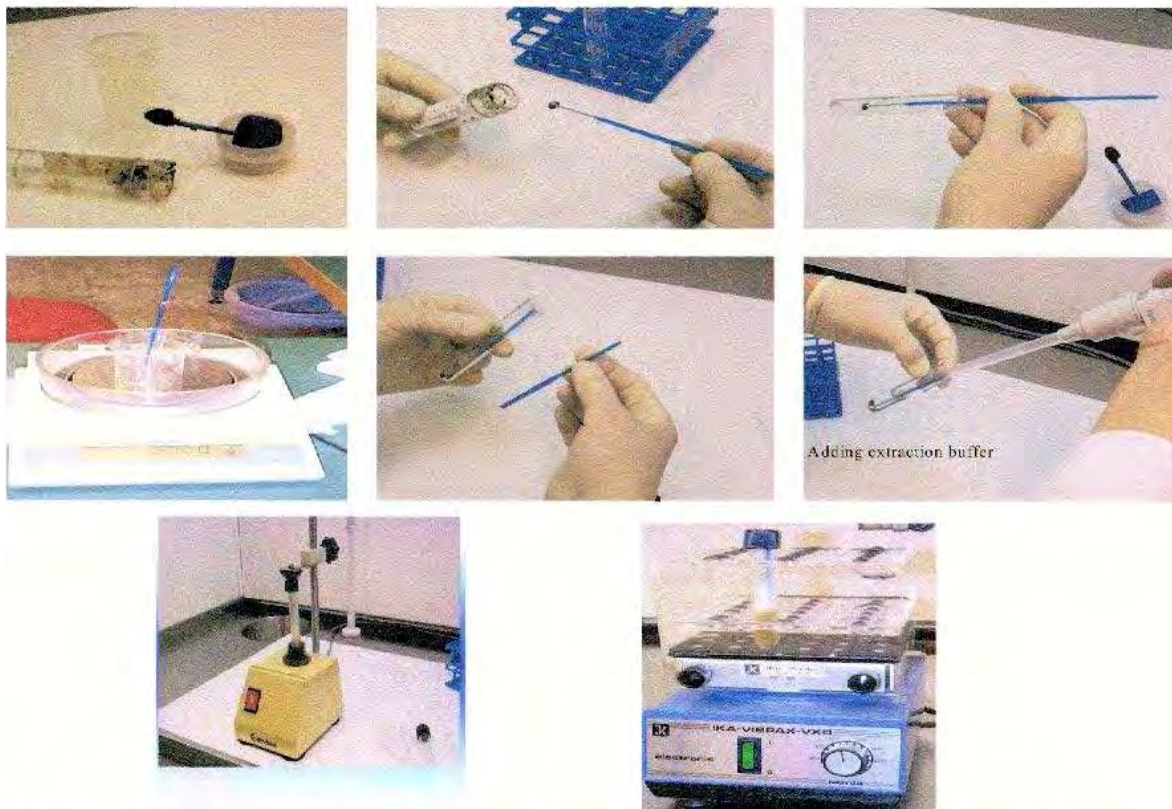
**Faeces:** Since calprotectin is very stable in stools, patients can collect small faecal samples at home. Collect 1 – 5 g (approximately one teaspoon full) and place it in a suitable container and deliver it to the laboratory within 4 days. When put in a transport container it can be sent by ordinary mail, i.e. no

refrigeration is needed. Samples can be stored frozen, at -18 °C or lower until delivery or mailing. Exposure to temperatures above 30 °C should be avoided. Before extraction frozen samples can be thawed at room temperature, for instance over night.

### **Practical steps:**

1. Weigh (tare) empty screw cap tube with the inoculation loop.
2. Take out approx. 100 mg (between 40 and 120 mg) faeces by means of the inoculation loop and place it into the screw cap tube. Avoid taking out solid, undigested material like fibres and seeds.
3. Weigh tube and loop with faeces which will give the net faeces weight.
4. Break off the top half of the loop handle and leave the bottom part inside the tube.
5. Add diluted extraction buffer to a weight/volume ratio 1:50, for instance 4.9 ml to 100 mg faeces. Close the tube.
6. Shake or mix vigorously for 30 seconds by means of a vortex mixer.
7. Continue the mixing on a shaker (at approx. 1000 rpm) for 30 +/- 5 minutes with the loop inside the tube as an agitator.
8. Transfer 1 – 2 ml of the homogenate to an Eppendorf tube and centrifuge at 10.000g for 20 min. at +4 °C for 20 min.
9. The clear supernatant is the extract to be diluted and run on the ELISA. About 0.5 ml is transferred to a new tube for assay or storage. Avoid contact with the pellet as aggregates or particles can cause erroneous calprotectin values. Extracts can be stored at +4 °C for several days or frozen up to 12 months.
10. The extracts are diluted 1:50 (20 µl sample + 980 µl dilution buffer) before running. Samples with very high concentrations may need re-testing after further dilution, for instance 1:5.

For easy handling and homogenizing of the samples the Sample preparation kit supplied by Roche Diagnostics in Mannheim (Art. Nr.: 745804) can be used.



**Collection and extraction of stool samples for the CALPRO Calprotectin ELISA Test**

## 8. Suggested plate configuration:

	1	2	3	4	5	6
A	Standard H 1000 ng/ml*	Standard H 1000 ng/ml*	Control	Control	Sample 8	Sample 8
B	Standard G 500 ng/ml	Standard G 500 ng/ml	Sample 1	Sample 1	Sample 9	Sample 9
C	Standard F 250 ng/ml	Standard F 250 ng/ml	Sample 2	Sample 2	Sample 10	Sample 10
D	Standard E 125 ng/ml	Standard E 125 ng/ml	Sample 3	Sample 3	Sample 11	Sample 11
E	Standard D 62.5 ng/ml	Standard D 62.5 ng/ml	Sample 4	Sample 4	Sample 12	Sample 12
F	Standard C 31.3 ng/ml	Standard C 31.3 ng/ml	Sample 5	Sample 5	Sample 13	Sample 13
G	Standard B 15.6 ng/ml	Standard B 15.6 ng/ml	Sample 6	Sample 6	Sample 14	Sample 14
H	Standard A 7.8 ng/ml	Standard A 7.8 ng/ml	Sample 7	Sample 7	Sample 15	Sample 15

\*optional standard

## 9. ASSAY PROCEDURE

Please read the test protocol carefully **before** performing the assay. Result reliability depends on strict adherence to the test protocol as described. Prior to commencing the assay, the distribution and identification plan for all specimens and controls should be carefully established on the result sheet supplied in the kit. Select the required number of microtiter strips or wells and insert them into the holder.

The standard H 1000 ng/ml is optional and applied if required.

Perform all assay steps in the order given and without any appreciable delays between the steps.

A clean, disposable tip should be used for dispensing each control and sample.

It is recommended to determine controls and patient samples in duplicate.

Allow all reagents to reach room temperature (18...25°C)

Thaw frozen samples at room temperature.

1. Dilute samples 1:50 (20 µl sample + 980 µl dilution buffer) and mix well.
2. Add 50 µl of each standard, control and diluted sample in duplicate wells in rows, see template.
3. Cover the plate with sealing foil and incubate at room temperature on a horizontal shaker for 45 +/- 5 min, 1000 rpm.
4. At the end of the incubation time, remove the liquid and wash the wells by adding 250 µl washing buffer to each well. Remove as much liquid as possible. Repeat these steps until a total of 5 washings have been performed. Check that aspirating or filling probes are not blocked so that the washing of some wells are inefficient. After the final aspiration, invert the plate and tap the well openings gently on absorbent tissue to remove remaining washing solution.
5. Mix content of vial gently prior to use (do not shake). Add 50 µl conjugate to each well.
6. Cover plate with sealing foil and incubate as above on a horizontal shaker (45 +/- 5 min at room temperature, 1000 rpm).
7. Repeat washing steps as above (5x 250 µl).
8. Add 100 µl substrate solution to each well preferably using a Repetitive Pipettor.
9. Incubate plate at room temperature for approx. 20 – 30 minutes in the dark.
10. Add 100 µl stop solution to each well.
11. Read the O.D. values by means of an ELISA reader at 405 nm.

## 10. QUALITY CONTROL

A new standard curve must be included in each run. The Control must be included in each run.

O.D. value of the 1000 ng/ml standard should be about 2.0. If a blank or 0 standard is used, its O.D. value should be below 0.2. Readings can be postponed for up to 24 hours if the plate is stored at +4°C.

## 11. Evaluation

The concentration of calprotectin in stools should be expressed as mg/kg.

The calculation of concentrations in patient samples can be performed by a computer linked to an ELISA reader, or manually as follows:

Calculate the mean optical densities (O.D.) of all duplicates. Plot the log values of the standard concentrations against their OD to obtain a standard curve. When using a computer program a 4-parameter or Spline function is recommended. Use this to find the diluted sample concentrations from their O.D. values. The reading of the control should be within the limits printed on the vial label.

**The values of the diluted samples are corrected for the dilutions and converted to mg/kg by multiplying by 2.5 (e.g.: a reading of 100 ng/ml becomes 250 mg/kg). If samples have been diluted further the additional dilution factor must be entered into the calculation.**

Calculation example:

**Value: 100 ng/ml equals 100 x 2,5 = 250 mg/kg**

## 12. INTERPRETATION OF RESULTS

In studies following values have been determined:

Normal value	5 – 50 mg/kg
Positive value	> 50 mg/kg
median value in patients with colorectal cancers	350 mg/kg.
active, symptomatic inflammatory bowel disease	200 - 20 000 mg/kg.

## 13. PRECISION

<b>Interassay</b>	<b>n</b>	<b>mean</b>	<b>CV [%]</b>
Positive Sera	2 (24)	0.89	5.1

<b>Intraassay</b>	<b>n</b>	<b>means</b>	<b>CV [%]</b>
Positive Sera	16	0.90	2.1

## 14. CLINICAL VALUATION

Comparison with the reference method measured by the Ullevaal University shows an agreement of  $r^2 = 0.976$ .

## 15. LIMITATIONS OF THE PROCEDURE

Repeated freeze-thaw cycles of the specimen may affect the absorbance values. Diagnosis of an infectious disease should not be established on the basis of a single test result. A precise diagnosis should take into consideration clinical history, symptoms as well as serological data.

## 16. PRECAUTIONS AND WARNINGS

- In compliance with article 1 paragraph 2b European directive 98/79/EC the use of the in vitro diagnostic medical devices is intended by the manufacturer to secure suitability, performances and safety of the product. Therefore the test procedure, the information, the precautions and warnings in the instructions for use have to be strictly followed. The use of the test kits with analyzers and similar equipment has to be validated. Any change in design, composition and test procedure as well as for any use in combination with other products not approved by the manufacturer is not authorized; the user himself is responsible for such changes. The manufacturer is not liable for false results and incidents for these reasons. The manufacturer is not liable for any results by visual analysis of the patient samples.
- Only for in-vitro diagnostic use.

- All components of human origin used for the production of these reagents have been tested for anti-HIV antibodies, anti-HCV antibodies and hepatitis B and have been found to be non-reactive. Nevertheless, all materials should still be regarded and handled as potentially infectious.
- Do not interchange reagents or strips of different production lots.
- No reagents of other manufacturers should be used along with reagents of this test kit.
- Do not use reagents after expiry date stated on the label.
- Use only clean pipette tips, dispensers, and lab ware.
- Do not interchange screw caps of reagent vials to avoid cross-contamination.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
- After first opening and subsequent storage check conjugate and control vials for microbial contamination prior to further use.
- To avoid cross-contamination and falsely elevated results, pipette patient samples and dispense conjugate without splashing accurately to the bottom of wells.
- The reagents included contain sodium azide at lower than 0.1 % (w/v). Sodium azide may react with lead and copper plumbing and form explosive salts. Always dispose of sodium azide containing material with large quantities of water.
- Avoid skin contact with the substrate. The solution should be pale yellow, stored in the dark, and shaken before use.
- The CALPRO Calprotectin ELISA is only designed for qualified personnel who are familiar with good laboratory practice.

<p><b>WARNING:</b> Sodium hydroxide causes severe burns. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing, gloves and eye/face protection. In case of accident or if you feel unwell, seek medical advice immediately.</p>
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## 16.1. Disposal Considerations

Residues of chemicals and preparations are generally considered as hazardous waste. The disposal of this kind of waste is regulated through national and regional laws and regulations. Contact your local authorities or waste management companies which will give advice on how to dispose hazardous waste.

## 17. REFERENCES

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## 18. ORDER INFORMATION

Product code: CAL0100      CALPRO Calprotectin ELISA Test (ALP) (96 Determinations)

Symbols Key/ Symbolschlüssel/ Explication des symboles / Legenda / Símbolos	
	In Vitro Diagnostic Medical Device/ In Vitro Diagnosticum/ Dispositif médical de diagnostic <i>in vitro</i> / Diganostico <i>in vitro</i> / Producto para diagnóstico In vitro
	Lot Number/ Chargenbezeichnung/ Numéro de lot/ Lotto/ Número de lote
	Expiration Date/ Verfallsdatum/ Date de péremption/ Scadenza/ Fecha de caducidad
	Storage Temperature/ Lagertemperatur/ Température de conservation/ Temperatura di conservazione / Temperatura de almacenamiento
	CE Mark/ CE-Zeichen/ Marquage CE / Marchio CE/ MarcaCE
	Catalogue Number/ Katalog Nummer/ Référence du catalogue/ Numero di codice/ Número de Catálogo
	Contains sufficient for "n" tests/ Ausreichend für "n" Tests/ Contenu suffisant pour "n" tests/ Contenido suficiente per "n" saggi/ Contenido suficiente para "n" tests

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