

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 the 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 according to the published methods (e.g., Johne 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 neutrophilic 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 of PMNs (Fagerhol et al., 1990) and can reliably be estimated in faecal samples stored for up to 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 (Johne 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 (Røseth et al., 1999), 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 in both children and adults, a negative result, as measured by the **CALPRO Calprotectin ELISA Test (ALP)** can save many endoscopies and thereby cost.

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 (ALP)** 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 usually positive in active IBD. The concentration of Calprotectin measured in stools is a non-invasive and objective marker; it can be used to determine disease activity and response to treatment of IBD, and to tell when a true

remission has been achieved. Many IBD patients in clinical remission, with normal clinical indices and normal CRP 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 (ALP)** is based upon preparation of an extract of about 0.1 gram faeces mixed with about 5 ml of Faecal Extraction Buffer in a closed tube. After centrifugation, a sample from the supernatant is tested by an enzyme immunoassay specific for Calprotectin.

The immunoassay requires 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 is determined by incubation with a substrate for the enzyme.

The rabbit antibodies used in the **CALPRO Calprotectin ELISA Test (ALP)** react with a number of different epitopes on Calprotectin, ensuring 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 (ALP)** is run on stool extracts prepared by the use of a patented Faecal 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.

4. MATERIALS

4.1. Reagents supplied with the kit

- **Coated microplate:** 12 strips, 8 wells per strip, coated with immunoaffinity-purified polyclonal rabbit antibodies specific for Calprotectin. The plate is stored in a sealed bag with desiccant.
- **Sample Diluent (5x conc.)^{*}:** 20 ml 5x concentrate, to be diluted with distilled water; pH 8.0 ± 0.2, yellow coloured solution, bottle with blue cap.
- **Washing Solution (20x conc.)^{**}:** 2 x 50 ml 20x concentrate, to be diluted with distilled water for washing the microtiter wells; pH 8.0 ± 0.2, clear solution, bottles with white caps.
- **Fecal Extraction Buffer (2.5x conc.)^{***}:** 2 x 90 ml 2.5x concentrate, to be diluted with distilled water; pH 8.0 ± 0.2, clear solution, bottles with a white caps.
- **Enzyme Conjugate^{****}** 8 ml alkaline phosphatase-labelled, immunoaffinity-purified polyclonal rabbit antibodies against Calprotectin; red coloured solution, opaque bottle with black cap.
- **Enzyme substrate solution (pNPP):** 13 ml, ready to use; clear to faint yellow solution, opaque bottle with yellow cap.
- **Calprotectin Standards^{*},** 8 vials with 0.6 ml, ready to use; yellow coloured solution, bottles with red caps:

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^{*},** 0.6 ml, ready to use; yellow coloured solution, bottle with green cap.
 - ^{*} Contains 0.1 % Kathon and <0.1 % sodium azide
 - ^{**} Contains 0.1 % Kathon
 - ^{***} Contains <0.1% sodium azide
 - ^{****} Contains 0.02% methylisothiazolone and 0.02% bromonitrodioxane

4.2. Materials supplied

- 2 Sealing foils
- 1 Test protocol
- 1 Plate layout

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 – 150 mg).
- Plate shaker (500 – 700 rpm).
- Microcentrifuge (10,000g)
- Freezer (-20°C or lower)
- Repetitive Pipettor or multi-channel pipette, 50 – 250 µl.
- Timer
- Microplate reader, filter 405 nm.
- Microplate well washer or multi-channel pipette, 50 – 250 µl.
- Pipettes to deliver volumes 10 – 1000 µl
- Vortex mixer.
- Disposable polystyrene screw cap tubes, 14 ml
- Distilled water
- 1 M NaOH (Stop Solution; optional)

5. STABILITY AND STORAGE

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

Opened plates, reagents and concentrated buffers are stable for up to three months when stored at 2 – 8°C.

Working solutions of Washing Solution, Sample Diluent and Extraction Buffer, prepared in clean vessels, can be stored at 2 – 8°C for one month. Avoid exposure to high temperature and direct sunlight.

6. REAGENT PREPARATION

It is 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 snap-off strips are coated with immunoaffinity-purified polyclonal rabbit antibodies specific for Calprotectin. Unused strips should be resealed in the aluminium foil pouch along with the desiccant supplied immediately after removal of strips, and stored at 2 – 8°C.

6.2. Enzyme-conjugated antibody

The bottle contains 8 ml of a solution of alkaline phosphatase (ALP)-labelled, immunoaffinity-purified rabbit antibodies against Calprotectin in a buffer with stabilizers, preservatives and an inert red dye. The solution is ready to use.

6.3. Standards and Control

The vials labelled with Standard A – H, as well as the Control, contain 0.6 ml each of a ready-to-use solution. The concentration of Calprotectin in the different vials is printed on the label.

6.4. Faecal Extraction Buffer

Dilute the concentrated Faecal Extraction Buffer by adding 1 part (90 ml) to 1.5 parts (135 ml) distilled water in a clean vessel to obtain 225 ml working solution. Mix well. Store the diluted buffer in a closed vessel at 2 – 8°C.

6.5. Washing Solution

Dilute the concentrated Washing Solution by adding 1 part (50 ml) to 19 parts (950 ml) distilled water in a clean vessel to a final volume of 1000 ml. Store the diluted Washing Solution in a closed vessel at 2 – 8°C.

6.6. Sample Diluent

Dilute the concentrated Sample Diluent by adding 1 part (20 ml) to 4 parts (80 ml) distilled water in a clean vessel to a final volume of 100 ml. Store the diluted Sample Diluent in a closed vessel at 2 – 8°C.

6.7. Enzyme Substrate Solution (pNPP)

The bottle contains 13 ml of *p*-nitrophenylphosphate (pNPP) solution. The solution is ready to use and must be stored in its original, opaque bottle.

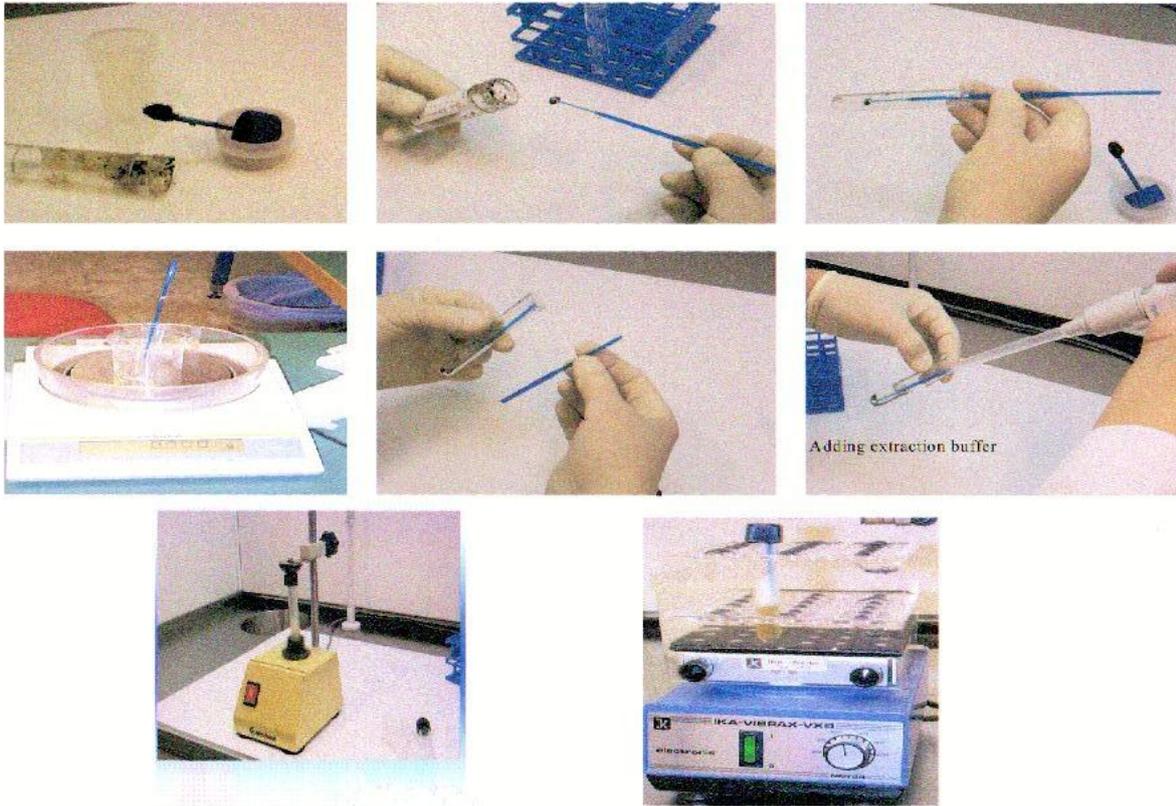
7. SPECIMEN COLLECTION AND PREPARATION

Since Calprotectin is very stable in stools, patients can collect small faecal samples at home. Collect 1 – 5 g (approximately one teaspoonful), place it in a suitable clean container and deliver it to the laboratory as soon as possible but within 4 days. When put in a container approved for transport, it can be sent by ordinary mail, i.e. no refrigeration is needed. Samples can also be stored frozen, at -20°C or lower, until delivery or mailing. Exposure to temperatures above 30°C should be avoided. Frozen samples must be thawed and equilibrated to room temperature before extraction and testing. Avoid freezing and thawing more than once.

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 fibers 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 Faecal Extraction Buffer to a weight: volume ratio 1:50, for instance 4.9 ml buffer to 100 mg faeces. Close the tube.
6. 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. The extract, which represents a 1:50 dilution of the stool sample, is now ready for dilution/testing. Allow a couple of minutes on the bench for particles to settle and pipette carefully from the top of the tube. No centrifugation is necessary, but a short centrifugation (for example at 5000 rpm in a bench top centrifuge) can be performed if a particle-free solution is required.
9. Transfer about 0.5 mL to a new tube for assay or storage. Extracts can be stored at 2 – 8°C for at least five days or frozen below -20°C for up to six months¹⁵⁾.

As an alternative to the above procedure, for easy handling and homogenising of faecal samples, the Faecal Sample Preparation kit sold separately by Calpro AS, Norway (Art. CAL0500) can be used.



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

8. SUGGESTED PLATE LAYOUT

	1	2	3	4	5	6
A	Standard H 1000 ng/mL*	Standard H 1000 ng/mL*	Control	Control	Sample8	Sample8
B	Standard G 500 ng/mL	Standard G 500 ng/mL	Sample 1	Sample1	Sample9	Sample9
C	Standard F 250 ng/mL	Standard F 250 ng/mL	Sample 2	Sample2	Sample10	Sample10
D	Standard E 125 ng/mL	Standard E 125 ng/mL	Sample 3	Sample3	Sample11	Sample11
E	Standard D 62.5 ng/mL	Standard D 62.5 ng/mL	Sample 4	Sample4	Sample12	Sample12
F	Standard C 31.3 ng/mL	Standard C 31.3 ng/mL	Sample 5	Sample5	Sample13	Sample13
G	Standard B 15.6 ng/mL	Standard B 15.6 ng/mL	Sample 6	Sample6	Sample14	Sample14
H	Standard A 7.8 ng/mL	Standard A 7.8 ng/mL	Sample 7	Sample7	Sample15	Sample15

*Optional standard (For use as a quality control measure for the assay); see Section 9

9. ASSAY PROCEDURE

Procedural Notes

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 Plate Layout in Section 8 for all specimens and controls should be carefully established using for example the sheet supplied in the kit. Select the required number of microtiter strips. Unused strips should be stored as described in Section 6.1.

Standard H (1000 ng/mL) is included in the test kit, but needs only to be included in order to ensure that the highest OD of the assay is reached after 20-30 min. incubation with the substrate. The highest precision and accuracy of the assay is >15.6 and <500 ng/mL (dynamic range) due to the high degree of linearity in this range. Standards A to G must be run in each assay. See Section 10, Quality Control.

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

A clean, disposable pipette tip should be used for dispensing each Standard, Control and sample.

To achieve the most reliable results, standards, controls and patient samples should always be run in duplicate.

All samples and kit reagents must be equilibrated to room temperature (18 – 25°C) for at least one hour before testing is begun.

ELISA Procedure

1. Dilute the faecal extracts 1:50 (20 µl sample + 980 µl Sample Diluent) and mix well. Extracts with Calprotectin values > 500 ng/mL should be further diluted, for instance 1:10 if an accurate value is needed
2. Add 50 µl of each Standard, Control and diluted sample in duplicate wells in rows; see recommended Plate Layout in Section 8.
3. Cover the plate with a sealing foil and incubate at room temperature on a horizontal plate shaker for 45±5 min at 500 – 700 rpm.
4. At the end of the incubation time, remove the liquid and wash the wells by adding 250 µl Washing Solution to each well. Remove as much liquid as possible. Repeat these steps until a total of 5 washings have been performed. If using a plate washer, check that all aspirating and filling probes are not blocked to ensure efficient washing of all wells. After the final wash, invert the plate and tap the well openings on absorbent tissue to remove any remaining Washing Solution.

5. Mix content of the Enzyme Conjugate vial gently prior to use (do not shake). Add 50 µl of conjugate to each well, preferably using a repetitive pipettor or a multichannel pipette.
6. Cover plate with sealing foil and incubate as above on a horizontal plate shaker 45±5 min at 500 – 700 rpm.
7. Repeat washing steps as above 5 times with 250 µl of Washing Solution.
8. Add 100 µl Enzyme Substrate Solution to each well, preferably using a repetitive pipettor or a multichannel pipette.
9. Incubate plate at room temperature for approx. 20 – 30 minutes, protected from light.
10. Add 100 µL 1M NaOH stop solution to each well if a fixed incubation period is always used by the laboratory.
11. Read the optical density (OD) values at 405 nm using an ELISA reader.

10. QUALITY CONTROL

A new standard curve must be included in each run. The Control must be included in each run. If included, the OD value of Standard H (1000 ng/mL) should be ≥ 2.0 . The OD value for Standard G (500 ng/mL) should be ≥ 1.5 . If a blank is used, its OD value should be below 0.2.

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 OD of all duplicates. Plot the log values of the standard concentrations against their mean OD values to obtain a standard curve. When using a computer program a 4-parameter function is recommended. Use this to determine the Calprotectin concentration in the diluted samples based on their OD values. The value of the Control should be within the limits printed on the vial label.

The values of faecal samples are corrected for the total dilution of 1:2500 (1:50 during the extraction procedure and 1:50 dilution of the extracts) and converted to mg/kg by multiplying with 2.5 (e.g. if a diluted sample has a value of 100 ng/mL, the concentration in the original stool specimen becomes $100 \times 2.5 = 250$ mg/kg). If samples have been diluted further the additional dilution factor must be entered into the calculation expressed in ng/mL

12. INTERPRETATION OF RESULTS

The following Calprotectin values in stool samples measured by the Calpro Calprotectin ELISA Test (ALP) have been reported (Johne et al., 2001; Røseth et al., 1992) in the published literature:

Normal value	5 – 50 mg/kg
Positive value	> 50 mg/kg
Median value in patients with symptomatic colorectal cancers	350 mg/kg
Active, symptomatic inflammatory bowel disease	200 – 40,000 mg/kg.

13. PRECISION

Typical Inter-assay Precision

The variation between repeated analyses of faecal samples within the dynamic range of the assay, were determined over 5 days, with 2 runs per day.

Range of Sample Tested	Low	Medium	High
Mean (ng/mL)	22	166	519
CV (%)	4.3	4.6	5.3
Range (ng/mL)	20-23	156-176	467-548
N	10	10	10

Typical Intra-assay Precision

The variation between repeated measurements of faecal samples within the dynamic range of the assay, in one assay, was determined.

Range of Sample Tested	Low	Medium	High
Mean (ng/mL)	24	155	463
CV (%)	8.7	6.9	6.1
Range (ng/mL)	21-28	139-173	423-522
N	20	20	20

14. CLINICAL EVALUATION

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

15. LIMITATIONS OF THE PROCEDURE

Repeated freeze-thaw cycles of the specimen may affect the accuracy of the test results. Diagnosis should not be established based on a single test result. Diagnosis should take into consideration clinical history and symptoms.

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 antigen (Bag) and have been found to be non-reactive. Nevertheless, all materials should be regarded and handled as potentially infectious.
- Do not interchange reagents or strips of different production lots.
- Do not use reagents from other manufacturers with reagents of this test kit.
- Do not use reagents after expiry date stated on the label or after 1 month of preparation of concentrated reagents to working solutions.
- Use only clean pipette tips, dispensers, and lab ware.
- To prevent cross contamination, do not interchange screw caps of reagent vials.
- Close reagent vials tightly immediately after use to avoid evaporation and microbial contamination.
- After first opening and subsequent storage, check conjugate, standards and Control vials for microbial contamination prior to further use.
- To avoid cross-contamination and falsely elevated results, pipette Standards, Control and faecal extract samples, and dispense conjugate and substrate, accurately to the bottom of microplate wells, without splashing..
- Some reagents contain sodium azide at less than 0.1% (w/v).
- Store the substrate solution in the original, opaque bottle; the solution should be clear to pale yellow. Mix gently before use.
- The CALPRO Calprotectin ELISA test (ALP) is designed for use by qualified personnel who are trained in good laboratory practice.

WARNING:	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.
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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/ Contenuto sufficiente per "n" saggi/ Contenido suficiente para "n" tests

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