ELISA (ALP) RUO

CALP0170RUO

LIST OF CONTENT

1 INTENDED USE

The **CALPROLAB™ ELISA (ALP)** is a quantitative method for the determination of Calprotectin in human biological samples.

For Research Use Only

2 BACKGROUND

Calprotectin is a 24 kDa dimer[1] formed by the monomers S100A8 (10,835 Da) and S100A9 (13,242 Da)[2,3]. Calprotectin has a high affinity for calcium, zinc[4], produced by PMNs, monocytes and squamous epithelial cells (except those in normal skin)[56]. After binding of calcium, it can resist degradation by leukocytic and microbial enzymes[5,7]. By competing with different enzymes[8] for limited, local amounts of zinc, Calprotectin can inhibit many zinc-dependent enzymes and thereby kill microorganisms or animal and human cells in culture)[9,10].

Many studies have shown elevation of Calprotectin in various bodily fluids, including serum/plasma, CSF, saliva, urine, synovial fluid and feces[4], and have been shown to be elevated in various acute and chronic conditions, such as various rheumatic conditions and diseases, inflammatory bowel disease (IBD), SLE, various cancers, sepsis, and other bacterial infections and acute and chronic inflammations

3 PRINCIPLE OF THE TEST

The **CALPROLAB™ (ALP) RUO** is based on principles of enzyme-linked immunoassay (ELISA). The assay has a sandwich ELISA setup, with mAb specific for human calprotectin as capture/coating and enzyme labelled rabbit pAb as secondary antibody. Standards and samples are incubated in coated wells to allow capture of calprotectin, and then second incubation is performed with enzyme-labelled pAb after repeated washing steps. Labelled pAb allows fo detection by color change of substrate, which can be measured at specific wavelengths with ELISA plate reader. Color change is proportional to calprotectin concentration in the sample. The assay is calibrated using native Calprotectin purified from a human leukocyte extract.

4 MATERIALS

4.1 Reagents and materials components supplied with the kit

- MTP **Coated microtiterplate:** 12 strips, 8 wells per strip, coated with affinity-purified monoclonal mouse antibodies specific for Calprotectin. The plate is stored in a sealed bag with desiccant.
- DIL 5x **Sample Dilution Buffer (5x conc.) ***:** 1 x 20 mL, 5x concentrate, to be diluted with distilled/deionised water; pH 8.0 ± 0.2, yellow coloured solution, bottle with blue cap.
- WASH BUF 20x **Washing Solution (20x conc.) *:** 1 x 50 mL, 20x concentrate, to be diluted with distilled/deionised water, for washing the microtiter wells; pH 7.8 ± 0.2, clear solution, bottle with white cap.
- FEC EXTR BUF 2,5x **Faecal Extraction Buffer (2.5x conc.) **:** 2 x 90 mL, 2.5x concentrate, to be diluted with distilled/deionised water; pH 8.0 ± 0.2, clear solution, bottles with white caps.
- CAL A - F **Calprotectin Standards ***:** 6 vials with 1.0 mL, ready to use; yellow coloured solution, vials with different coloured caps.

Standard:	A	B	C	D	E	F
Cap color:	Blue	Green	Yellow	Red	White	Black
Conc.:	0 ng/mL	7,8 ng/mL	31,3 ng/mL	62,5 ng/mL	125 ng/mL	500 ng/mL

- CTR LOW CTR HIGH **Calprotectin Controls "Low" and "High" ***:** 2 vials with 1.0 mL, ready to use; yellow coloured solution; Ctr Low: vial with brown cap;.Ctr High: vial with purple cap.
- CONJ **Enzyme Conjugate ****:** 13 mL alkaline phosphatase-labelled, immunoaffinity-purified polyclonal rabbit antibodies against Calprotectin, ready to use; red coloured solution, 25 mL Dynex reagent tube with white cap.
- SUB pNPP **Enzyme Substrate Solution (pNPP):** 13 mL**,** ready to use; clear to faint yellow solution, opaque bottle with yellow cap.
  Note: If using a Dynex instrument, the substrate has to be transferred into a 25mL Dynex reagent tube before running the test.

* Contains 0.1 % Kathon

** Contains <0.1% sodium azide

*** Contains 0.1 % Kathon and <0.1% sodium azide

**** Contains 0.02% methylisothiazolone and 0.02% bromonitrodioxane

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

4.2 Materials and equipment required but not supplied

- Distilled/deionised water
- Vortex mixer
- Disposable tubes for dilution of samples: Eppendorf tubes or similar (if assay is performed manually)
- Pipettes to deliver volumes 10 – 1000 µL (if assay is performed manually)
- Repetitive pipette or multi-channel pipette, 100 µL (if assay is performed manually)
- Microplate well washer or multi-channel pipette, 300 µL (if assay is performed manually)
- Plate shaker (500 – 700 rpm) (if assay is performed manually)
- Timer (if assay is performed manually)
- Microplate reader, filter 405 nm (if assay is performed manually)
- 1M NaOH (stop solution; optional)

5 STABILITY AND STORAGE

When stored unopened at 2 – 8°C, kit reagents are stable up to the expiry date stated on the label.

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

When prepared in clean vessels, working solutions (1x) of Washing Solution and Sample Dilution Buffer can be stored at 2 – 8°C for up to one month.

6 PREPARATION OF COMPONENTS

All reagents, samples and controls should be brought to room temperature (RT) (18 – 25°C) before starting the test run. All test components should be stored closed and at 2 – 8°C to ensure stability. Standards, controls, enyme conjugates and enzyme substrate solution (pNNP) are supplied as ready-to-use solutions. pNNP is also light sensitive and should be stored in its original opaque bottle.

Dilution of the remaining components are highlighted below.

6.1 Sample Dilution Buffer

Dilute the 5x concentrated Sample Dilution Buffer by adding 1 part (20 mL) to 4 parts (80 mL) distilled/deionised water in a clean vessel to a final volume of 100 mL. Mix well.

Note: If using a Dynex DS2 or DSX ELISA automat, the Sample Dilution Buffer must be transferred to a 25 mL Dynex reagent tube before running the test.

6.2 Faecal Extraction buffer

If extracting fecal samples, dilute the 2.5x concentrated Faecal Extraction Buffer by adding 1 part (90 mL) to 1.5 parts (135 mL) distilled/deionised water in a clean vessel to a final volume of 225 mL. Mix well.

6.3 Washing Solution

Dilute the 20x concentrated Washing Solution by adding 1 part (50 mL) to 19 parts (950 mL) distilled/deionised water in a clean vessel to a final volume of 1000 mL. Mix well.

7 PROCEDURE

7.1 Sample collection and preparations

For matrix specific sample preparations, please read Chapter 8

7.2 ELISA protocol (manual)

Precautions:

- Perform all steps in order without any unnecessary delays
- Use clean and single-use pipette tips when handling the components and samples
- Samples are recommended in duplicates for more reliable results
- Allow samples and reagents to equilibrate to RT (18-25°C) before use

ELISA Procedure

1. Prepare reagents (chapter 6) and sample dilutions (chapter 8)
2. Pipette 100 µL of standards and controls (in duplicate) and samples into designated wells.

   Incubate with sealing foil for 40±5 min at RT with gentle continuous shaking (~500 rpm).
3. Aspirate and wash 3 times with 300 µL of 1x**Washing solution** per well. Ensure to remove all liquid after each wash. Following the final wash, invert and tap on absorbent paper to remove excess liquid.
4. Add 100 µL of the ready-to-use **Enzyme conjugate** into each well. Incubate with sealing foil for

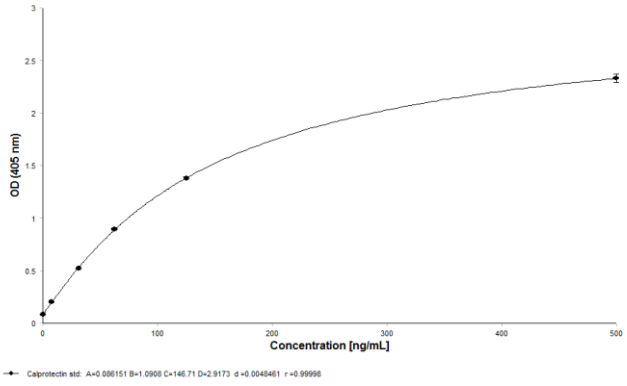
   40±5 min at RT with gentle continuous shaking (~500 rpm).
5. Aspirate and wash 3 times with 300 µL of 1x**Washing solution** per well. Ensure to remove all liquid after each wash. Following the final wash, invert and tap on absorbent paper to remove excess liquid.
6. Add 100 µL of the ready-to-use **Enzyme Substrate Solution** to each well. Incubate with sealing foil for 20-30 min without shaking. Cover the plate to block out light.
7. *Optional:* Add 100 µL 1M NaOH stop solution to each well if a fixed incubation period is required.
8. Read the absorbance at 405 nm. If the plate reader has this option, shake the plate briefly (2-3 seconds) before reading.

NOTE:

- Incubation time in step 2 and 4 can be reduced to 30±5 min without affecting the results[46]; However, this will result in OD<1,8 for the highest standard (recommended).
- It is possible to run this assay on automated instruments. Calpro can provide protocols/setups for certain instruments (e.g. Dynex DS2).

7.3 Quality control

- A new standard curve and the positive control must be included in each run.
- The value of the positive control should be within the limits printed on the vial labels.
- As a guide, the OD of Standard F (500 ng/mL) should be ≥1.6 (preferably ≥1,8), while the OD of Standard A (0 ng/mL) should be ≤0,25. A representative standard curve is shown to the right.



7.4 EVALUATION

1. Calculate the mean OD_{405} of all duplicate standards and draw a calibration curve based on these. It is recommended to use a 4-parameter curve fit function. If logarithmic scale is required, the value of the Standard A (blank) must be replaced with 0,001 ng/mL.
2. Calculate the concentration of the controls to ensure their average OD_{405} is within the specified range – when the controls are within the range, the curve can be used for samples.
3. Calculate the concentration of the samples using the approved calibration curve. If samples are used in *5000* duplicates, then the average OD of the wells are to be used to identify the concentrations, and results are provided in ng/mL.
4. Multiply the results from the curve with appropriate factor according to the dilution of the initial sample to obtain the correct value (see Chapter 8 and respective subcategories)
5. Use the calibration curve to determine the Calprotectin concentration in the diluted samples (ng/mL) based on their OD values.

8 SAMPLE HANDLING AND PREPARATION

8.1 Fecal samples

8.1.1 Sample collection

- Only small amount of faecal sample is needed for the analysis, but it is recommended that at least 5g sample is collected, equivalent to a teaspoon.
- Calprotectin in faecal sample is stable for up to 4 days at RT and several months at -20^{o}C (latter can result in somewhat elevated levels of Calprotectin). Avoid exposing the samples to high temperatures (>30^{o}C) for prolonged periods of time.
- Sample can be collected by the patient at home and sent/dropped off at the lab within reasonable time (point above).
- Patient can also use EasyExtract™ (CAL0510, Calpro) sample collection and extraction device. For details, refer to the EasyExtract™ IFU.

8.1.2 Sample extraction and preparation

It is recommended to use the EasyExtract™ (CAL0510) extraction device for preparing faecal extract. For method outline, please refer to its manual. The kit is supplied with extra extraction buffer in case other methods of extraction is being used.

8.1.3 Running the faecal extract on CalproLab RUO

- Allow the extract to equilibrate to RT before use
- Make a 1:100 dilution of the faecal extract (Step 8.1.2) in 1xSample Dilution Buffer
- Add 100µl of the working solution to the CalproLab ELISA RUO wells and follow the procedure as outlined in Chapter 7 Procedure.
- Once OD_{405} is read and concentration in ng/mL is determined according to the calibration curve, the final concentration is calculated using following calculation:

$$\frac{Result\ from\ calibration\ curve \times dilution\ factor\ of\ extract \times dilution\ factor\ in\ sample\ dilution\ buffer)}{1000} = [mg/kg]$$

If extract and dilution in sample dilution buffer is prepared according to the protocol (EasyExtract and further 1:100 dilution), the concentration from calibration curve will be multiplied by 5 (50x100/1000) to give sample concentration in mg/kg (µg/g).

8.1.4 Reference range

Based on internal studies and scientific publications the normal range for healthy individuals are:

Sample type:	**Negative results**
faecal samples	<50 mg/kg

8.1.5 Storage stability

	20-25°C	2-8°C	-20°C
Fecal sample:	4 days		
Extract with EasyExtract	5 days	7 days	2 years

8.1.6 Range, linear range, LoQ, LoD, and LoB

Recommended final dilution in dilution buffer:	1:5000 (Extract: 1:50, further 1:100 dilution in dilution buffer)
Range of assay at recommended dilution:	25-2500 mg/kg
LoQ	22,15 mg/kg
LoD	4,36 mg/kg
LoB	0,71 mg/kg

8.1.7 Repeatability

sample type	**calprotectin conc.-**	**Repeatability (%CV)**	**within laboratory precision (%CV)**
Faecal extract	32 mg/kg	7.6	14.6
Faecal extract	171 mg/kg	4.4	7.1
Faecal extract	363 mg/kg	4.4	10.1
Faecal extract	557 mg/kg	3.8	6.0
Faecal extract	1086 mg/kg	7.7	14.0
Faecal extract	1715 mg/kg	9.8	9.6

8.2 Plasma/EDTA samples

8.2.1 Sample collection

- Collect blood using suitable blood collection equipment treated with EDTA.
- Centrifuge the collected sample at 3000 rpm for 10 minutes as soon as possible and no later than three hours after sampling.
- Harvest only the upper two thirds of the plasma layer by careful pipetting so that leucocytes from the buffy coat are not aspirated.

8.2.2 Running the plasma/EDTA on CalproLab RUO

- Allow the plasma/EDTA to equilibrate to RT before use
- Create a final dilution by making 1:50 dilution of the sample in 1x Sample Dilution Buffer
- Add 100µl of the working solution to the CalproLab ELISA RUO wells and follow the procedure as outlined in Chapter 7 Procedure.
- Once OD_{405} is read and concentration in ng/mL is determined aaccording to the calibration curve, the final concentration is calculated using following calculation:

$$Result\ from\ calibration\ curve \times dilution\ factor\ in\ sample\ dilution\ buffer\ = [ng/mL]$$

If extract and working dilution is prepared according to the protocol above, the concentration from calibration curve will be multiplied by 50 to give sample concentration in ng/mL.

8.2.3 Range, linear range, LoQ, LoD, and LoB

Recommended working dilution:	1:50
Range of assay at recommended dilution:	410-8000 ng/mL
Linear range of assay at recommended dilution:	178,9-7977.4 ng/mL
LoQ	410 ng/mL
LoD	343 ng/mL
LoB	127 ng/ml

8.2.4 Repeatability

sample type	**calprotectin conc.-**	**Repeatability (%CV)**	**within laboratory precision (%CV)**
EDTA plasma	849 ng/mL	5.8	8.2
EDTA plasma	5926 ng/mL	4.2	5.1

9 LIMITATIONS OF THE PROCEDURE

Caution is advised when using samples spiked with purified calprotectin samples. Due to matrix effects in some samples recovery of spiked protein is not linear.

10 PRECAUTIONS AND WARNINGS

- Research use only.
- 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 months 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) and/or 0.1% Kathon.
- Store the substrate solution in the original, opaque bottle; the solution should be clear to pale yellow. Mix gently before use.
- The **CALPROLAB™ ELISA RUO** is designed for use by qualified personnel who are trained in good laboratory practice.

11 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.

12 ORDERING INFORMATION

Product code: CALP0170ROU

Product name: CALPROLAB™ ELISA RUO

13 REFERENCES

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14 LEGENDS USED ON PACKAGING

Symbols Key / Symbolschlüssel / Tabela de símbolos	
	Manufactured by / Hergestellt von / Fabricado por / Fabricado por
LOT	Lot Number / Chargenbezeichnung / Número de lote
	Expiration Date / Verfallsdatum / Data de Validade
	Storage Temperature / Lagertemperatur / Temperatura de almacenamiento
REF	Catalogue Number / Katalog Nummer / Número de Catálogo
	Catalogue Number / Katalog Nummer / Número de Catálogo
MTP	Microplate / Mikrotiterplatte / Microplaca
CONJ	Conjugate / Konjugat / Conjugado
CAL	Calibrator A-F / Kalibrator A-F / Calibrador A-F
CTR LOW	Control Low / Kontrolle Niedrig / Control Bajo
CTR HIGH	Control High / Kontrolle Hoch / Control Alto
DIL 5x	Sample diluent buffer 5x concentrated / Probenverdünnungspuffer 5x konzentriert / Solución tampón para muestras concentrado x5
SUB pNPP	pNPP Substrate solution / pNPP-Substratlösung / Solución substrato pNPP
FEC EXTR BUF 2,5x	Faecal Extraction Buffer 2,5x concentrated / Stuhlextraktionspuffer 2,5x konzentriert / Buffer fecal de extracción (2,5 x conc.)
	Contains sufficient for “n” tests / Ausreichend für “n” Tests / Contenido suficiente para ”n” tests

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