



HK325

HUMAN CALPROTECTIN

ELISA KIT

PRODUCT INFORMATION & MANUAL

Read carefully prior to starting procedures!

ATTENTION

For use in laboratory research only
Not for clinical or diagnostic use

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 quality control 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 human calprotectin ELISA kit is to be used for the *in vitro* quantitative determination of human calprotectin in plasma, urine, faeces, and cell culture supernatant samples. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

The kit can not be used for quantification of the individual MRP-8 (S100A8) or MRP-14 (S100A9) proteins.

The analysis should be performed by trained laboratory professionals.

2. INTRODUCTION

Calprotectin, also known as MRP-8/MRP-14 or S100A8/A9 heterocomplex, is formed out of the calcium-binding, migration inhibitory factor-related proteins, MRP-8 (S100A8) and MRP-14 (S100A9). The expression of these proteins is largely confined to the cytosol of neutrophils and monocytes. The complex formation of these proteins is calcium-dependent. Calprotectin comprises 60% of the cytoplasmic protein fraction of circulating polymorphonuclear granulocytes and is also found in monocytes, macrophages and ileal tissue eosinophils. Peripheral blood monocytes carry the antigen extra- and intracellularly, neutrophils only intracellularly. Calprotectin has antibacterial, antifungal, immunomodulating and antiproliferative effects. Furthermore, it is a potent chemotactic factor for neutrophils. Plasma concentrations are elevated in diseases associated with increased neutrophil activity. During intestinal wall inflammation, granulocytes transmigrate through the intestinal wall. Therefore calprotectin is also detectable in faeces. Several investigations report that faecal calprotectin is significantly increased in intestinal diseases such as inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis and colon cancer.

Normal human plasma contains a calprotectin concentration ranging from 500 to 3000 ng/ml.

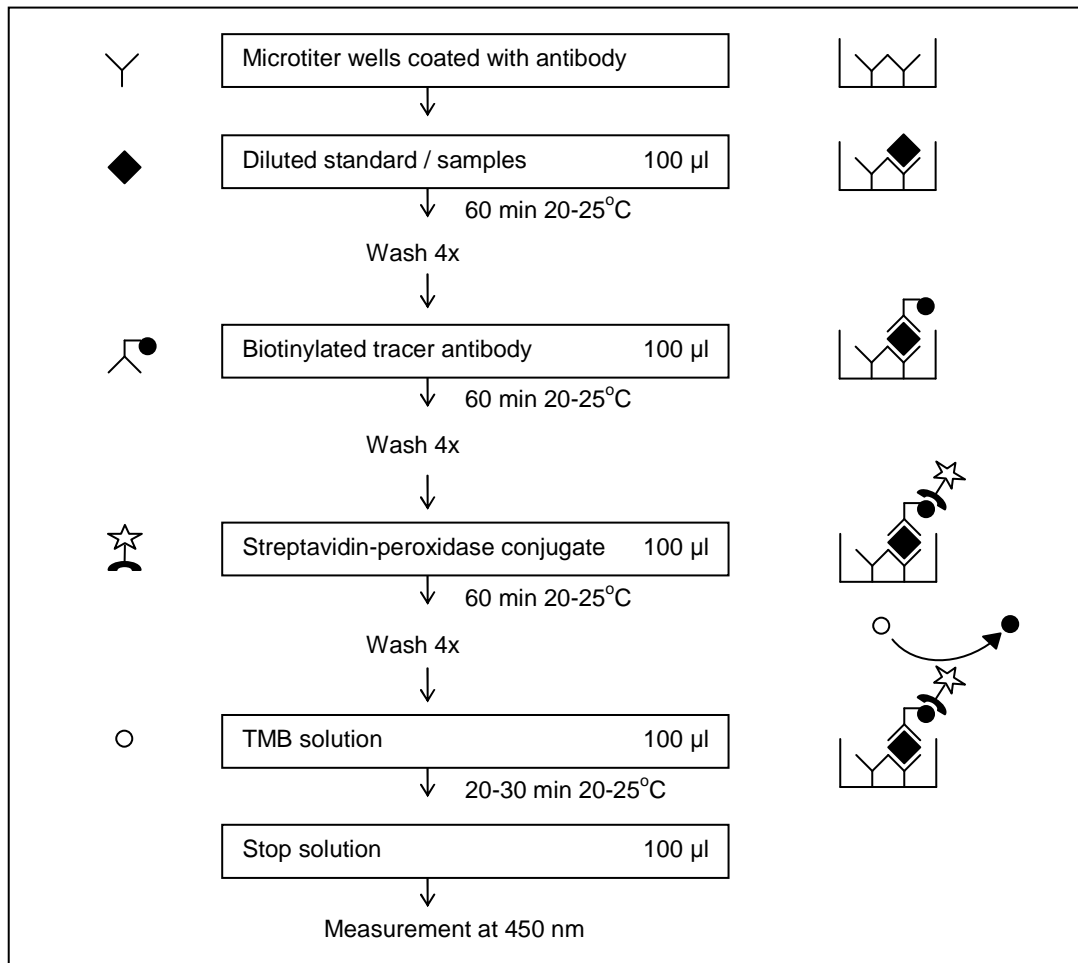
3. KIT FEATURES

- Working time of 3½ hours.
- Minimum concentration which can be measured is 1.6 ng/ml.
- Measurable concentration range of 1.6 to 100 ng/ml.
- Working volume of 100 µl/well.

Cross-reactivity

Cross-reactivity for other species or proteins/peptides has not been tested.

4. PROTOCOL OVERVIEW



- The human calprotectin ELISA is a ready-to-use solid-phase enzyme-linked immunosorbent assay based on the sandwich principle with a working time of 3½ hours.
- The efficient format of 2 plates with twelve disposable 8-well strips allows free choice of batch size for the assay.
- Samples and standards are incubated in micro titer wells coated with antibodies recognizing human calprotectin.
- Biotinylated tracer antibody will bind to captured human calprotectin.
- Streptavidin-peroxidase conjugate will bind to the biotinylated tracer antibody.
- Streptavidin-peroxidase conjugate 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 human calprotectin standards (log).
- The human calprotectin concentration of samples, which are run concurrently with the standards, can be determined from the standard curve.

5. KIT COMPONENTS AND STORAGE INSTRUCTIONS

Item no.	Kit component	Quantity	Color code
Vial 1	Wash buffer 40x	1 vial (20 ml)	Grey
Vial 2A	Dilution buffer A 10x	1 vial (10 ml)	Gold
Vial 2B	Dilution buffer B 10x	1 vial (10 ml)	Gold
Vial 3	Standard	2 vials, 0.5 ml lyophilized	Yellow
Vial 4	Tracer, biotinylated	2 vials, 1 ml lyophilized	Green
Vial 5	Streptavidin-peroxidase	1 vial, 1 ml lyophilized	Blue
Vial 6	TMB substrate	1 vial (20 ml)	Purple
Vial 7	Stop solution	1 vial (20 ml)	Red
Item 8	12 Microtiter strips, pre-coated	2 plates	
Item 9	Frame	1	
Item 10	Adhesive covers	4	
Item 11	Certificate of quality control	1	
Item 12	Manual	1	
Item 13	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.
- The standard, tracer and streptavidin-peroxidase are stable in lyophilized form until the expiration date indicated on the kit label, if stored at 2 - 8°C.
- The exact concentration of the standard is indicated on the label of the vial and the certificate of quality control.
- After reconstitution the standard must be used within 15 minutes. For longer stability we recommend to store aliquots at -20°C. Stored at -20 °C the standard will be stable for 1 month.
- Once reconstituted, tracer and streptavidin-peroxidase are stable for 1 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 guaranteed until expiration date if stored at 2 - 8°C.

Materials required but not provided

- Calibrated micropipettes and disposable tips.
- Distilled or de-ionized water.
- Plate washer: automatic or manual.
In case a plate washer is used the supplied wash buffer is not sufficient. Additional wash buffer can be ordered separately. Please contact your local distributor.
- Polypropylene tubes.
- Calibrated ELISA plate reader capable of measuring absorbance at 450 nm.

6. WARNINGS AND PRECAUTIONS

- For research use only, not for diagnostic or therapeutic use.
- This kit should only be used by qualified laboratory staff.
- Do not under any circumstances add sodium azide as preservative to any of the components.
- 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 manufacturer.
- The assay has been optimized for the indicated standard range.
- Standard, tracer and streptavidin-peroxidase vials should be opened after reconstitution. Open vials carefully: vials are under vacuum.
- Do not ingest any of the kit components.
- Kit reagents contain 2-chloroacetamide as a preservative. 2-Chloroacetamide is harmful in contact with skin and toxic if swallowed. In case of accident or if you feel unwell, seek medical advice immediately.
- The TMB substrate is light sensitive, keep away from bright light. The solution should be colorless until use.
- The stop solution contains 2% oxalic acid and can cause irritation or burns to respiratory system, skin and eyes. Direct contact with skin and eyes should be strictly avoided. If contact occurs, rinse immediately with plenty of water and seek medical advice.
- Incubation times, incubation temperature and pipetting volumes other than those specified may give erroneous results.
- Do not reuse micro wells or pour reagents back into their bottles once dispensed.
- Handle all biological samples as potentially hazardous and capable of transmitting diseases.
- 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.

7. SAMPLE PREPARATION

Collection and handling

Plasma

Please be aware that human calprotectin is released from neutrophils into serum in the process of blood coagulation. This will lead to false positive results. It is therefore advised to use 'careful plasma', which can be obtained as follows.

Keep freshly collected blood on ice. Within 20 minutes after blood sampling, separate plasma by centrifugation: 1500xg at 4°C for 15 min. Remove plasma and transfer to fresh polypropylene tube. Be careful to not disturb white cells in the buffy coat. Recentrifuge the transferred plasma in order to avoid every contamination with white blood cells: 1500xg at 4°C for 15 min.

Note that most reliable results are obtained with EDTA plasma.

Urine

Collect urine using normal aseptic techniques. Centrifuge the urine to remove debris (1500xg at 4 °C for 15 min). Transfer urine to a fresh poly propylene tube.

Faeces

Calprotectin can be measured in faeces if samples are extracted using the following extraction buffer: 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 5 ml extraction buffer to 100 mg sample (giving a dilution factor of 51, assuming the density of faeces to be 1 g/ml). Vortex samples and filter the samples to remove coarse particles (> 0.6 mm). Shake the filtrate for 20 minutes and centrifuge samples: 10,000xg at 4°C for 20 minutes. Use supernatant for analysis.

Storage

Store samples below -20°C, preferably at -70°C in polypropylene tubes. Storage at -20°C can affect recovery of human calprotectin. Use samples within 24 hours after thawing. Avoid multiple freeze-thaw cycles which may cause loss of human calprotectin activity and give erroneous results.

Do not use hemolyzed, hyperlipemic, heat-treated or contaminated samples.

Before performing the assay, 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

Plasma samples

Human calprotectin can be measured accurately if plasma samples are diluted at least 60x with supplied dilution buffer in polypropylene tubes.

Note that most reliable results are obtained with EDTA plasma.

Urine samples

Human calprotectin can be measured accurately if urine samples are diluted at least 10x with supplied dilution buffer in polypropylene tubes. For calprotectin detection in urine, the standard curve could be adapted to 0.6-40 ng/ml to increase sensitivity

Faeces samples

Human calprotectin can be measured accurately if extracted faeces samples are diluted with supplied dilution buffer in polypropylene tubes. It is recommended to test both 800x (A) and 1200x (B) diluted supernatant. Subsequently, determine the calprotectin-ratio by division of both calculated calprotectin-concentrations (B/A). When this ratio is > 1.2, the sample contains certain factors that interfere with reliable calprotectin-concentration determination

Remark regarding recommended sample dilution

The recommended dilution for samples should be used as a guideline. 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.

Do not use polystyrene tubes or sample plates for preparation or dilution of the samples.

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 20 ml of 40x wash buffer with 780 ml of distilled or de-ionized water, which is sufficient for 2 x 96 tests. Where less volume is required, prepare the desired volume of wash buffer by diluting 1 part of the 40x wash buffer with 39 parts of distilled or de-ionized water.

Dilution buffer A and B

Prepare dilution buffer by mixing 10 ml of the 10x dilution buffer A and 10 ml of 10x dilution buffer B with 80 ml distilled or de-ionized water, which is sufficient for 2 x 96 tests. Where less volume is required, prepare the desired volume of dilution buffer by diluting 1 part of the 10x dilution buffer A and 1 part of 10x dilution buffer B with 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 solution

The standard is reconstituted by injection of 0.5 ml of distilled or de-ionized water. After reconstitution the standard must be used within 15 minutes. For longer stability we recommend to store aliquots at –20°C. Prepare each human calprotectin standard in polypropylene tubes by serial dilution of the reconstituted standard with dilution buffer as shown in Table 2.

Tube	Volume dilution buffer	Volume standard	Concentration (ng/ml)
1	See certificate of quality control	225 µl vial 3	100
2	225 µl	225 µl tube 1	50
3	225 µl	225 µl tube 2	25
4	225 µl	225 µl tube 3	12.5
5	225 µl	225 µl tube 4	6.3
6	225 µl	225 µl tube 5	3.1
7	225 µl	225 µl tube 6	1.6
8	225 µl	-	0

Table 2

Tracer solution

The tracer is reconstituted by injection of 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml tracer with 11 ml dilution buffer, which is sufficient for 1 x 96 tests. Where less volume is required, prepare the desired volume of tracer by diluting 1 part of the reconstituted tracer with 11 parts of dilution buffer.

Streptavidin-peroxidase solution

The streptavidin-peroxidase is reconstituted by injection of 1 ml distilled or de-ionized water. Dilute the reconstituted 1 ml streptavidin-peroxidase with 23 ml dilution buffer, which is sufficient for 2 x 96 tests. Where less volume is desired, prepare the required volume of streptavidin-peroxidase solution by diluting 1 part of the reconstituted streptavidin-peroxidase with 23 parts of dilution buffer.

9. ELISA PROTOCOL

Bring all reagents to room temperature (20 - 25°C) before use.

1. Determine the number of test wells required, put the necessary micro well 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.
2. Transfer 100 µl in duplicate of standard, samples, or controls into appropriate wells. Do not touch the side or bottom of the wells.
3. Apply an adhesive cover to the tray. Tap the tray to eliminate any air bubbles. Be careful not to splash liquid onto the cover.
4. Incubate the strips or plate for 1 hour at room temperature.
5. Wash the plates 4 times with wash buffer using a plate washer or as follows:
 - a. Carefully remove the plate sealer, 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 5b.
 - d. Repeat the washing procedure 5b/5c three times.
 - e. Empty the plate and gently tap on thick layer of tissues.
6. Add 100 µl of diluted tracer to each well using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
7. Cover the tray with an adhesive cover. Incubate the tray for 1 hour at room temperature.
8. Repeat the wash procedure described in step 5.
9. Add 100 µl of diluted streptavidin-peroxidase to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
10. Cover the tray with an adhesive cover, incubate the tray for 1 hour at room temperature.
11. Repeat the wash procedure described in step 5.
12. Add 100 µl of TMB substrate to each well, using the same pipetting order as applied in step 2. Do not touch the side or bottom of the wells.
13. Cover the tray with a new adhesive cover, incubate the tray for 20 – 30 minutes at room temperature. Avoid exposing the micro well strips to direct sunlight. Covering the plate with aluminum foil is recommended.
14. Stop the reaction by adding 100 µl of stop solution with the same sequence and timing as used in step 12. Mix solutions in the wells thoroughly by gently swirling the plate. Gently tap the tray to eliminate any 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, following the instructions provided by the instrument's 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). For an example of the standard curve see certificate of quality control included with the kit. If the standard is out of range, the results of the test samples are not reliable. The test should be repeated.
- 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. If the standard is out of range, the results of the test samples are not reliable. The test should be repeated.
- 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, sample, tracer, streptavidin-peroxidase 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.
- To ensure accurate results, proper adhesion of supplied covers during incubation steps is necessary.
- The waste disposal should be performed according to your laboratory regulations.

Technical support

Do not hesitate to contact our technical support team at support@hycultbiotech.com for inquiries and technical support regarding the human calprotectin ELISA.

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12. QUALITY CONTROL

The certificate of quality control 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 is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Hycult Biotech immunoassay, the possibility of interference cannot be excluded.

For optimal performance of this kit, it is advised to work according to good laboratory practice.

13. 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 experimental data should be sent to support@hycultbiotech.com.

Suggestions summarized below in Table 3 can be used as a guideline in the 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 were brought to 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 micro titer reader
	•	•			Air bubbles
		•			Imprecise sealing of the plate after use
•					Wrong storage conditions

Table 3

14. REFERENCES

1. Peake, J et al; Systemic inflammatory responses to maximal versus submaximal lengthening contractions of the elbow flexors. *Exerc Immunol Rev* 2006, *12*: 72
2. Leclercq A et al; Involvement of intraplaque hemorrhage in atherothrombosis evolution via neutrophil protease enrichment. *J Leukoc Biol* 2007, *82*: 1420
3. Martin-Ventura J et al; Low plasma levels of HSP70 in patients with carotid atherosclerosis are associated with increased levels of proteolytic markers of neutrophil activation. *Atherosclerosis* 2007, *194*: 334
4. Peake, J et al; Body temperature and its effect on leukocyte mobilization, cytokines and markers of neutrophil activation during and after exercise. *Eur J Appl Physiol* 2008, *102*: 391
5. Gecse, K et al; Increased faecal serine protease activity in diarrhoeic IBS patients: a colonic luminal factor impairing colonic permeability and sensitivity. *Gut* 2009, *57*: 591