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(54) Title: ELISA FOR CALPROTECTIN

(57) Abstract: The present invention provides an improved method for the detection and quantification of calprotectin in a sample.



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Title: ELISA for calprotectin

Field of invention

The present invention concerns an improved Enzyme-Linked Immunosorbent Assay (ELISA) for detection of a protein or polypeptide, particularly calprotectin, in particular methods that allow selection of suitable antibodies.

Background of invention

Calprotectin belongs to the S100 family of proteins. The name derives from the fact that they are resistant to precipitation by ammonium sulphate so that they are soluble even in 100 per cent saturated (thus 100S) solution of ammonium sulphate.

It is believed that they have evolved by a large number point mutation, but many amino acid sequence homologies remain. For this reason, some antibodies can bind to epitopes that are common for many or at least several S100 proteins. A common feature of these proteins is that they can bind calcium and zinc and thereby become resistant to enzymatic degradation; this is especially true for calprotectin. In the presence of calcium calprotectin will form dimers, while S100A12 (hereafter called A12) will form oligomers, mostly dimers, tetramers and hexamers. Calprotectin is a heterotrimer consisting of two subunits called S100A9 (A9) and one called S100A8 (A8). It has been found that each of these subunits can bind two calcium molecules, i.e. a total of six per calprotectin molecule.

The subunits and their genes were fully sequenced in the late 1980's and thus are well known in the art. (Odink et al., "Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis", *Nature*, 1987 Nov 5-11; 330 (6143): 80-2 Lagasse et al., "cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation," *Mol. Cell Biol.* 1988 Jun; 8(6): 2402-10, Andersson et al., "The leukocyte L1 Protein: identity with the cystic fibrosis antigen and the calcium-binding MRP-8 and MRP-14 macrophage components, " *Scand. J. Immunol.*, Aug; 28(2): 241-5 (1988). Also the crystalline structures of the subunits have been determined, (Itou et al., "The crystal structure of human MRP14 (S100A9), a Ca(2+)-dependent regulator protein in inflammatory process." *J Mol. Biol.* 2002 Feb 15; 316(2):265-76, Itou et al., "Expression, purification, crystallization and preliminary X-ray diffraction analysis of human calcium-binding protein MRP14 (S100A9)". *Acta Crystallogr. D Biol.*

Crystallogr. 2001 Aug; 57(pt 8): 1174-6, Moncrief et al., "Evolution of EF-hand calcium-modulated protein. I. Relationship based on amino acid sequence," J. Mol. Evol., June; 30(6): 522-62 (1990), Raftery et al., "Overexpression, oxidative refolding, and zinc binding of recombinant forms of the murine S100 protein MRP14 (S100A9)," Protein Expr. Purif. 1999 Mar; 15(2): 228-35, Raftery et al.,
5 "Isolation of the murine S100 protein MRP14 (14 kDa migration-inhibitory-factor-related protein) from activated spleen cells: characterization of post-translation modifications and zinc binding," Biochem. J., May 15; 316 (Pt1): 285-93 (1996), Loomans et al., "Histidine-based zinc-binding sequence and the antimicrobial
10 activity of calprotectin," J. Infect. Dis. Mar; 177(3): 812-4 (1998), Rety et al., "Structural basis of the Ca(2+)-dependent association between S100C (S100A11) and its targets, the N-terminal part of annexin I," Structure Fold. Des. 2000 Feb 15; 8(2): 175-84.

Both calprotectin and S100A12 are abundant in neutrophil granulocytes and
15 monocytes and are released from these cells during inflammation, cell damage or cell death. They are therefore found in increased concentration in blood, other body fluids, secretions and excretions during inflammation for which they may be useful markers.

Calprotectin may be used as a marker for a number of diseases wherein excessive
20 levels of calprotectin activity characterise the diseases. Such diseases include, but are not limited to, inflammatory bowel disease, rheumatoid arthritis, cystic fibrosis, inflammatory dermatosis, liver diseases, neurodegenerative diseases, Alzheimer's disease, dementia, multiple sclerosis and cancers.

Calprotectin has commonly been used as a marker to distinguish between organic
25 and functional gastrointestinal disease and for the early diagnosis of inflammatory bowel disease. It is a useful marker for screening of inflammatory bowel disease, for assessment of disease activity and response to treatment and for determining the prognosis of inflammatory bowel disease in patients already diagnosed with this disorder. Normalization of faecal calprotectin can be reliably be used to determine
30 that mucosal healing, the ultimate goal of treatment, has been achieved. Hence the most practical and specific biomarker for the diagnosis, detection, or monitoring of inflammatory bowel disease is calprotectin. A number of assays for calprotectin detection and quantification are already known, however, there is a need for an

improved assay for calprotectin that is specific for calprotectin and has a wide dynamic range. Structural differences between calprotectin in stool extracts and the calibrators may lead to false results; in particular, falsely low values will be found in immunoassays if calprotectin in stool extracts is lacking epitopes that are well
5 expressed in the calibrators. The present invention also presents a method for selection of antibodies to avoid this problem.

Summary of invention

Using S100A9 as calibrators and highly selected anti-S100A9 antibodies, one monoclonal and one rabbit polyclonal, reproducible and accurate estimates for
10 calprotectin can be achieved. The antibodies are selected on the basis that they react exclusively with S100A9 epitopes that are present on calprotectin in stool extracts. The invention also includes the use of citrate buffer as a coating buffer on a solid support, for instance microplate wells in an ELISA format. The monoclonal is used for coating while the polyclonal antibody is used as a conjugate for detection of
15 calprotectin bound to the coat. For conjugation different substances or particles can be used, but typically an enzyme like alkaline phosphatase or horseradish peroxidase are preferred. The ELISA procedure comprises 1) providing a suitable monoclonal anti-S100A9, 2) coating of the monoclonal antibody onto microwells, 3) incubation of calibrators/sample in separate wells to allow calprotectin to bind to
20 the monoclonal antibody in the coat, 4) washing out non-bound substances, 5) incubation in the wells with the antibody conjugate, 6) washing out non-bound antibodies, 7) incubation with a substrate for the enzyme in the conjugate, 8) reading the signal, i.e. the colour intensity resulting from conversion of the substrate, being proportional to the concentration of calprotectin in the sample.

Brief description of the drawings

Figure 1 is a standard curve for specific S100A8 ELISA using monoclonal antibodies.

Figure 2 shows a standard curve obtained when using a single specific S100A9 monoclonal antibody for coating of wells and a polyclonal antibody enzyme
30 conjugate.

Figure 3 is a standard curve for an ELISA using one monoclonal antibody both for coating of wells and as enzyme conjugate.

Detailed description of invention

The present invention involves an improved ELISA for the detection and quantification of calprotectin in a biological sample, and a diagnostic kit using the method.

Both calprotectin and S100A12 are abundant in neutrophil granulocytes and monocytes and are released from these cells during inflammation, cell damage or cell death. They are therefore found in increased concentration in blood, other body fluids, secretions and excretions during inflammation for which they may be useful markers. If for some reason one of them is not elevated, the other may still be. It might therefore be advantageous to use both markers simultaneously, or select antibodies that react with conserved amino acid sequences that are common for the two proteins. Out of thirteen monoclonal antibodies generated by immunization of mice with calprotectin, two showed a strong cross-reaction with S100A12. Among 10 rabbits immunized with calprotectin, four produced antibodies that cross-reacted with S100A12.

It is believed that calcium binding leads to a conformational change of calprotectin so that mostly hydrophobic parts of the molecule will be found on its surface. This may have at least two consequences for immunoassays of calprotectin:

- a) hydrophilic epitopes may not be available for binding to some antibodies;
- b) the hydrophobic parts may bind to other hydrophobic substances in the gut lumen to form complexes where calprotectin epitopes may be hidden or altered.

The original calprotectin ELISA was developed by Magne Fagerhol, MD at Ullevaal University Hospital, Oslo, Norway (herein after abbreviated as UUH). Antibodies were raised by immunization of rabbits with native calprotectin purified from extracts of neutrophil granulocytes. The rabbits were not selected in any way other than being the standard type used by the Animal department of the hospital. IgG fractions were isolated from mixtures of sera from 10 rabbits by use of ammonium sulphate precipitation and DEAE ion exchange chromatography. Subsequently, anti-calprotectin was purified by immunoaffinity chromatography using a column with 30 mg calprotectin covalently bound to sepharose beads. When that animal department was closed down, another source was sought. First a source

in Sweden was tried. Their rabbits produced antibodies (hereinafter abbreviated as PAbS) giving very good results in the sense that the shape of standard curves and assay range were much better giving an upper limit eight times the original.

5 However, when stool extracts were tested on the original and new ELISA, the latter gave only 25 to 50 per cent of the former. Out of this came the idea that the number and structure of epitopes on calprotectin in the standard and the extracts might differ significantly, and that some antisera might contain antibodies reacting better with the former. The reasoning was partly based upon previous results from running stool extracts on gel permeation chromatography (GPC) showing that calprotectin
10 eluted in high molecular weight (MW) fractions corresponding to 100 to 1000 kDa or larger while native calprotectin has a MW of 36 to 72 kDa. At the time extracts were prepared by homogenization of small stool samples in phosphate buffered saline; by repeated extraction of the same portion, it was found that the yield was only about 15 % in the first extract. When a new extraction procedure including a
15 more sophisticated extraction buffer was developed, the yield increased to about 50 %; when such extracts were run of the GPC a significant, but variable proportion of the calprotectin eluted in fractions corresponding to the MW of native protein. It was hypothesized that important antigenic epitopes on calprotectin in stool extracts might be hidden in complexes or have an altered structure as a consequence of
20 altered molecular configuration of calprotectin caused by factors in the gut content. The latter would be more likely to occur in conformational as opposed to linear epitopes.

The epitopes of protein or polypeptide antigens are divided into two categories, conformational epitopes and linear epitopes. The division is based on their structure
25 and interaction with the part of the antibody that recognises the antigen i.e. the paratope. A conformational epitope is composed of discontinuous sections of the antigen's primary amino acid sequence which are brought close to each other due to the secondary or tertiary structure of the antigen. Conformational epitopes interact with the paratope based on spatial shape or tertiary structure of the antigen.

30 On the other hand linear epitopes interact with the paratope based on their primary structure i.e. a continuous sequence of amino acids from the antigen.

An aspect here relates to the principle of quantitative immunoassays: to obtain a correct result the analyte in the sample should have the same structure and molecular configuration as that in the sample. Clearly this is very difficult to achieve when calprotectin in stool extracts is so heterogeneous.

- 5 To test the hypotheses mentioned above, recombinant calprotectin subunits were prepared, i.e. S100A8 (abbreviated as A8) and S100A9 (abbreviated as A9) which spontaneously form heterodimers (here called recombinant calprotectin) when the subunits are incubated with low concentrations, typically 2 mM, calcium chloride. These proteins, as well as recombinant S100A12, a closely related protein, were
- 10 coated in microwells which allowed testing of different antisera and purified antibodies to determine their reaction spectra. Furthermore, immunoaffinity columns were prepared by coupling S100A8, S100A9 and recombinant calprotectin in addition to the native calprotectin column, so that binding and non-binding antibodies could be tested.
- 15 It was found that the original antibody, from UUH, reacted only against calprotectin, recombinant calprotectin and S100A9. In contrast, the PAbS reacted also against S100A8. Part of that reactivity remained even if the antibodies were purified on a S100A9 affinity column suggesting that some antibodies reacted with epitopes that are common to S100A9 and S100A8. If epitopes on S100A8 are less
- 20 available on calprotectin in stools than in the standard, and anti-S100A8 contributes to signal in the ELISA, falsely low concentrations in samples must be expected. To test this hypothesis, a specific S100A8 ELISA using monoclonal antibodies was established giving the standard curve disclosed in figure 1.

- Thirteen stool extracts with calprotectin concentration between 21 and 817 ng/ml
- 25 were tested on this ELISA, but they all gave a signal clearly below that of the lower standard. This supports that antibodies reacting with S100A8 should be avoided in assays for calprotectin in stool extracts.

- It is therefore hypothesised that this problem could be solved by the use of only S100A9 as standard. This will be in agreement with the principle that for
- 30 quantitative immunoassays the analyte in the sample and standards should be in the same molecular conformation, i.e. if S100A8 is lacking in the extracts, it should

also be absent in the standards. At the present, this can only be achieved by use of recombinant S100A9.

In addition to the polyclonal rabbit antibodies, a series of mouse monoclonal antibodies (Mabs) were tested like the former. Since falsely low values in stool
5 extracts started to appear when antisera contain S100A8 epitopes were tried, it was chosen to start working with Mabs reacting with S100A9 only.

Another factor for the net ELISA result is the antibody used for preparation of antibody-enzyme conjugates. The ideal combination of coat and conjugate should give the correct result in stool extract when compared with the original UUH
10 method. A large number of different combinations of monoclonal antibodies and affinity purified polyclonal antibodies were therefore tried for coating or enzyme conjugation.

Surprisingly, excellent results were obtained when one particular monoclonal antibody was used for coating inadvertently in citrate buffer rather than the standard
15 carbonate buffer, together with an alkaline phosphatase immunoaffinity purified polyclonal antibody, i.e. the polyclonal antibody had been affinity purified on a native calprotectin column. For the first time similar values in stool samples as those with the original ELISA were found.

In one aspect of the invention a monoclonal antibody reacting with S100A9 is used.
20 Said monoclonal antibody is prepared by use of methods generally known in the art and wherein the antigen used is S100A9, preferably recombinant S100A9.

In another aspect the present invention provides sandwich immunoassays for S100A9 comprising the following steps:

- 1) preparing a surface of a solid support to which a known quantity of monoclonal
25 antibodies to said recombinant S100A9 is bound,
- 2) applying a sample containing calprotectin to the solid support prepared in step 1) binding calprotectin present in the sample to the monoclonal antibodies bound to the support,
- 3) washing of the solid support,
- 30 4) applying a polyclonal antibody against S100A9 to which a suitable marker, for instance an enzyme has been linked so that the labelled polyclonal antibody against S100A9 will bind to the calprotectin bound to the monoclonal antibodies on the

solid support,

5) washing of the solid support,

6) applying a method that can determine the amount of label bound to the solid support, for instance a substance that is converted into a detectable entity by the

5 labelling enzyme, and

7) measuring the detectable entity to determine the presence and quantity of calprotectin.

Suitable markers to be used in the method can be, but are not limited to, enzymes, gold particles, stained latex particles, or magnetic particles.

10 When the marker is an enzyme, enzyme labelled polyclonal antibody converts its substrate into a detectable entity being for instance a colour, fluorescence or electrochemical signal. The absorbance, fluorescence or electrochemical signal (e.g. current) of the detectable entity is measured by methods well known in the art, and the presence and quantity of calprotectin can be determined from the intensity of the
15 measured signal.

A recombinant S100A9 subunit for use as an antigen can be produced by expression of a corresponding DNA sequence by methods well known in the art in a suitable expression system. Both amino acid sequence and DNA sequence of S100A9 is as mentioned before well known to the skilled in art.

20 The S100A9 peptide may also be synthesised using all the known methods of chemical synthesis but particularly useful is the solid-phase methodology of Merrifield employing an automated peptide synthesiser (J. Am. Chem. Soc., 85: 2149 (1964)). The peptide may also be synthesised through solution peptide synthesis methods known in the art, either in a step-wise manner from the carboxyl
25 terminus and/or through the application of segment condensation or ligation methods, employing comprehensive or minimal protection strategies. Combined solution-solid phase segment condensation approaches can also be applied.

Such prepared and purified recombinant S100A9 or synthesised S100A9 peptide is then used in the preparation of monoclonal and polyclonal antibodies by standard
30 techniques well known in the art.

The ELISA assay according to the invention is a form of sandwich ELISA, and to facilitate the separation of the bound labelled reactant from excess non-bound

labelled reactant the non-labelled monoclonal antibody against S100A9 is attached to the surface of the solid support. The solid support may be, but is not limited to microtiter well, magnetic beads or plastic beads.

Several enzymes are known in the art as suitable for use as enzyme-label in an ELISA assay. Such enzymes include, but are not limited to, alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β -galactosidase. Additional enzyme labels are also known in the art. Such labels include, but are not limited to, acetate kinase, β -lactamase, glucose oxidase, firefly luciferase, laccase, Renilla luciferase, and xanthine oxidase. Alkaline phosphatase and horseradish peroxidase are usually preferred. The preparation of enzyme-labeled antibodies can be performed by methods known in the art.

The enzyme in the enzyme-labeled antibody generally converts its substrate into a product which is detectable and/or quantifiable photometrically, such as by spectroscopy, or detectable and/or quantifiable by fluorescence, bioluminescence, or electrochemical signaling.

In a particular embodiment of the invention the ELISA sandwich assay is performed, wherein the buffer used to coat the solid support with the monoclonal antibody is a citrate buffer. Typically, the citrate buffer is at a concentration of from about 50 mM to about 150 mM, preferably the citrate buffer concentration is from 75 mM to about 125 mM, more preferred the citrate buffer concentration is about 100 mM. Typically, the pH of the citrate buffer is from about pH 5 to about pH 7, preferably the pH is from about 5.5 to about 6.5, more preferred the pH is about 6.0. A particularly preferred buffer is 0.1 M sodium citrate, pH 6.0.

ELISA sandwich assays according to the present invention can be used for the detection and quantification of calprotectin in human biological materials like such as a fecal sample, a gastrointestinal tract sample, a blood sample e.g. a serum or plasma sample, a saliva sample a urine or a spinal fluid sample. It can also be used on biological samples from non-human species, for instance primates or domestic animals in which calprotectin shares epitope with that of human origin.

When the sample is a fecal sample or a gastrointestinal tract sample, the sample can be extracted prior to performance of the assay according to the procedure described in U.S. patent no. 6,225,072 or by any other suitable extraction buffer and/or procedure. This extraction procedure comprises: (1) mixing a small amount of sample (preferably 10 to 500 mg and more preferably 20-150 mg, optionally pre weighed) with an excess amount of aqueous extraction buffer (preferably in the region of a 50-fold excess (v/v)), comprising at least one dissociating,

disaggregating, and/or chelating agent; (2) homogenizing the sample (preferably by vortexing), in a closed tube; (3) separating the solid and liquid material of the dispersion resulting from homogenization of the sample (preferably by centrifugation and additionally or optionally by filtration); and (4) recovering the substantially clear liquid extract resulting from the separation, which contains calprotectin as well as other proteins. A suitable buffer is a citrate buffer with a pH of from about pH 5 to about pH 10. The citrate buffer can be the same citrate buffer described above. In addition to or in stead of citrate, other chelators could be used. The dissociating agent can be an agent such as polyoxyethylenesorbitan monolurate (Tween) or urea; urea concentrations up to 1 M are particularly suitable. Additionally, the buffer can contain 0.5% to 2% of bovine serum albumin (BSA), optionally in saline.

By use of extraction tubes containing a metal coil the dispergation and solubilization of calprotectin in the stool sample will be sufficient after vortexing for 3-5 minutes; in this case centrifugation can be omitted.

In another aspect of the invention a method is provided for the selection of antibodies that react adequately with calprotectin in stool extracts. In said method antibodies are screened against a panel of stool extracts from a large number of patients with active inflammatory bowel disease and the results are compared with those obtained by use of the original ELISA for calprotectin, the selected antibodies must give the same results as said original ELISA.

Generally, the assays according to the present invention, either purified calprotectin or purified S100A9 protein (e.g., recombinant S100A9) can be used as the standard. Often it is preferred to use purified S100A9 protein, particularly recombinant S100A9 protein, as the standard because purification of calprotectin from leukocytes is laborious, complex giving variable yields and often unstable protein. The invention is further illustrated by the following non-limiting examples and specific embodiments of the invention.

Examples:

Example 1

Coating of microwells: 96 well microplates with high protein binding capacity, for instance MaxiSorp, Nunc, Thermo Fischer Scientific International, can be used. For coating, to each well is added 100 to 200 μl , preferably 150 μl , of the highly selected monoclonal antibody (Mab), for instance the Calpro Mab CAL1-4H1/2/2, in a suitable concentration, for instance 1 to 4 $\mu\text{g/ml}$, preferable 2 $\mu\text{g/ml}$, in 0.1 M sodium citrate pH 6. The wells are covered by vapour tight adhesive plastic and stored at +4 centigrades for a suitable period of time, for instance six hours to several weeks, preferably 18 hours. The antibody coat can be stabilized by washing the wells once in PBS followed by addition of 200 μl /well of a suitable stabilizing solution, for instance StabilCoat from Surmodics in Vitro Diagnostic Products, Eden Prairie, MN, USA, for a suitable period of time, for instance 1-2 hours. For prolonged storage and/or shipment of the microplate, the wells can be washed 2-3 times with PBS, wells emptied and dried followed by covering the plate with an adhesive foil, wrapping it in a water vapour tight foil containing a water absorbent, for instance silica particles. Before use, excess antibodies are removed by washing each well three to four times with a suitable washing buffer instance 50 mM tris, 150 mM NaCl, 0.5 mM MgCl_2 , 2.5 mM KCl, 0.1 g/l thimerosal, 0.5 ml Tween-20 per liter, pH 8.0, To different wells are added 50 to 150 μl , preferably 100 μl , standards with known concentrations of calprotectin or S100A9 and samples in a suitable sample dilution buffer, for instance 50 mM tris, 150 mM NaCl, 0.5 mM MgCl_2 , 2.5 mM KCl, 0.1 g/l thimerosal, 1 % bovine serum albumin, 0.5 ml Tween-20 per liter, pH 8.0, and dilution. The wells are covered by tape or a lid and incubated at a suitable temperature, for instance room temperature for 10 to 60 minutes, preferably 40 minutes with horizontal shaking at about 1000 rpm. The wells are washed again as above, and to each well is added 50 to 150 μl , preferably 100 μl , of an enzyme conjugated highly selected immunoaffinity purified polyclonal antibody in a suitable buffer dilution, for instance the sample dilution buffer described above, and the plate is incubated again as above. The enzyme used for conjugation can be of any type suitable for immunoassays, for instance alkaline phosphatase. After washing again as above, 50 to 200 μl , preferably 100 μl , of a suitable substrate solution, for instance para-nitrophenylphosphate, is added to each well. The plate is left at room temperature for 10 to 60 minutes, preferably 30 minutes, after which the colour intensity in each well is measure by an ELISA

reader. The concentration of calprotectin in the samples is determined by comparison of the colour intensities in the respective wells with those of the standards taking the sample dilution factor into the calculation.

Figure 2 shows a typical standard curve from such an ELISA, and shows the standard curve obtained when using a single specific S100A9 Mab for coating of wells and a PAbS enzyme conjugate.

The differences between results when eight stool samples were tested on the calprotectin ELISA in Figures 2 and 3 are shown in Table I

Extract	Results, ng/ml	
	Using ELISA from Fig. 2	Using ELISA from Fig. 3
1	376	263
2	315	180
3	421	233
4	357	301
5	145	81
6	305	178
7	236	151
8	380	263
9	481	301
10	375	178

Clearly, the new ELISA using a combination of monoclonal and polyclonal antibodies gave higher values. The discrepancies were even higher for samples with concentrations of 500 ng/ml and above.

When testing samples with relatively low or normal levels of calprotectin, the two methods gave similar results, although there was a tendency for the pure monoclonal ELISA to give higher values. These results show that reliable estimates of calprotectin in stool extracts require carefully selected antibodies. Although the

monoclonal antibodies reacted with S100A9 epitopes, some of them were probably less available or absent on calprotectin in the extracts.

Example 2:

5 To check the possibility that S100A9 could be used as a standard instead of calprotectin, a series stool extracts were tested on the same ELISA as described above, see Fig. 2.

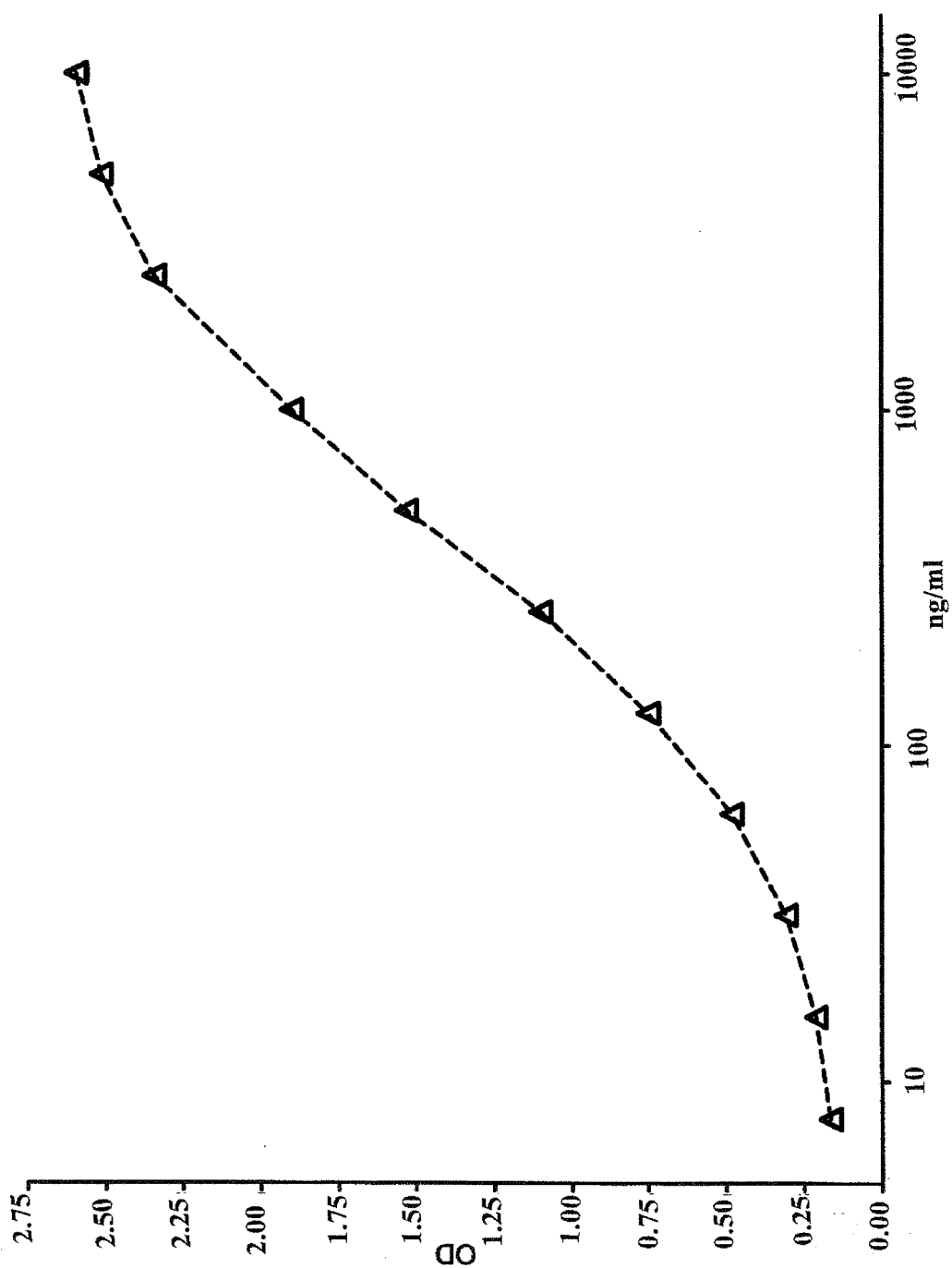
As shown in Fig. 4, an excellent correlation was found; the r^2 was 0.9936.

CLAIMS

1. Method for the detection and quantification of calprotectin in a sample comprising the steps:
 - 1) preparing a surface of a solid support to which a known quantity of monoclonal antibodies to said recombinant S100A9 is bound,
 - 2) applying a sample containing calprotectin to the solid support prepared in step 1) binding calprotectin present in the sample to the monoclonal antibodies bound to the support,
 - 3) washing of the solid support,
 - 4) applying a labelled polyclonal antibody against S100A9 binding the labelled polyclonal antibody against S100A9 to the calprotectin bound to the monoclonal antibodies on the solid support,
 - 5) washing of the solid support,
 - 6) applying a substance that is converted into a detectable entity by the labelling enzyme, and
 - 7) measuring the detectable entity to determine the presence and quantity of calprotectin.
2. Method according to claim 1, wherein the label of the polyclonal antibody is selected from the group consisting of enzymes, gold particles, stained particles, and magnetic particles, preferably an enzyme.
3. Method according to claim 2 wherein step 6 comprises the incubation of the solid support with a substrate for the enzyme of the enzyme-labelled polyclonal antibody wherein the substrate is enzymatically converted into a detectable entity.
4. Method according to claim 3, wherein the detectable entity is detected and/or quantified photometrically.
5. Method according to claim 4, wherein enzyme converted product is detected and/or quantified by a technique selected from the group consisting of fluorescence, bioluminescence.
6. Method according to claim 3, wherein the detectable entity is detected and/or quantified electrochemically.

7. Method according to any one of the preceding claims wherein the enzyme of the enzyme-labelled polyclonal antibody is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β -galactosidase.
- 5 8. Method according to claim 1, wherein a buffer is used when preparing the solid support with monoclonal antibodies and said buffer is a citrate buffer.
9. Method according to claim 8, wherein the concentration of the citrate buffer is from about 50 mM to about 150 mM, preferably from 75 mM to about 125 mM, more preferred about 100 mM and
- 10 wherein the pH of the citrate buffer is from about pH 5 to about pH 7, preferably from about 5.5 to about 6.5, more preferred about 6.0.
10. Method according to any one of the preceding claims, wherein the size of the sample is from about 50 μ L to about 150 μ L per assay or standard to be performed, preferably the size of the sample is about 100 μ L per assay or standard to be performed.
- 15 11. Method according to claim 10, wherein the sample is a fecal sample, a gastrointestinal tract sample, a blood sample, a serum sample, a plasma sample, a saliva sample, a urine sample, a spinal fluid sample or a biological sample from an animal in which calprotectin shares epitopes with the human protein
- 20 12. Method for the selection of antibodies that react adequately with calprotectin in stool extracts, wherein said antibodies are screened against a panel of stool extracts from a large number of patients with active inflammatory bowel disease and compare the results with those obtained by use of the original ELISA for
- 25 calprotectin, the selected antibodies must give the same results as said original ELISA.

Figure 1:
S100A8 ELISA



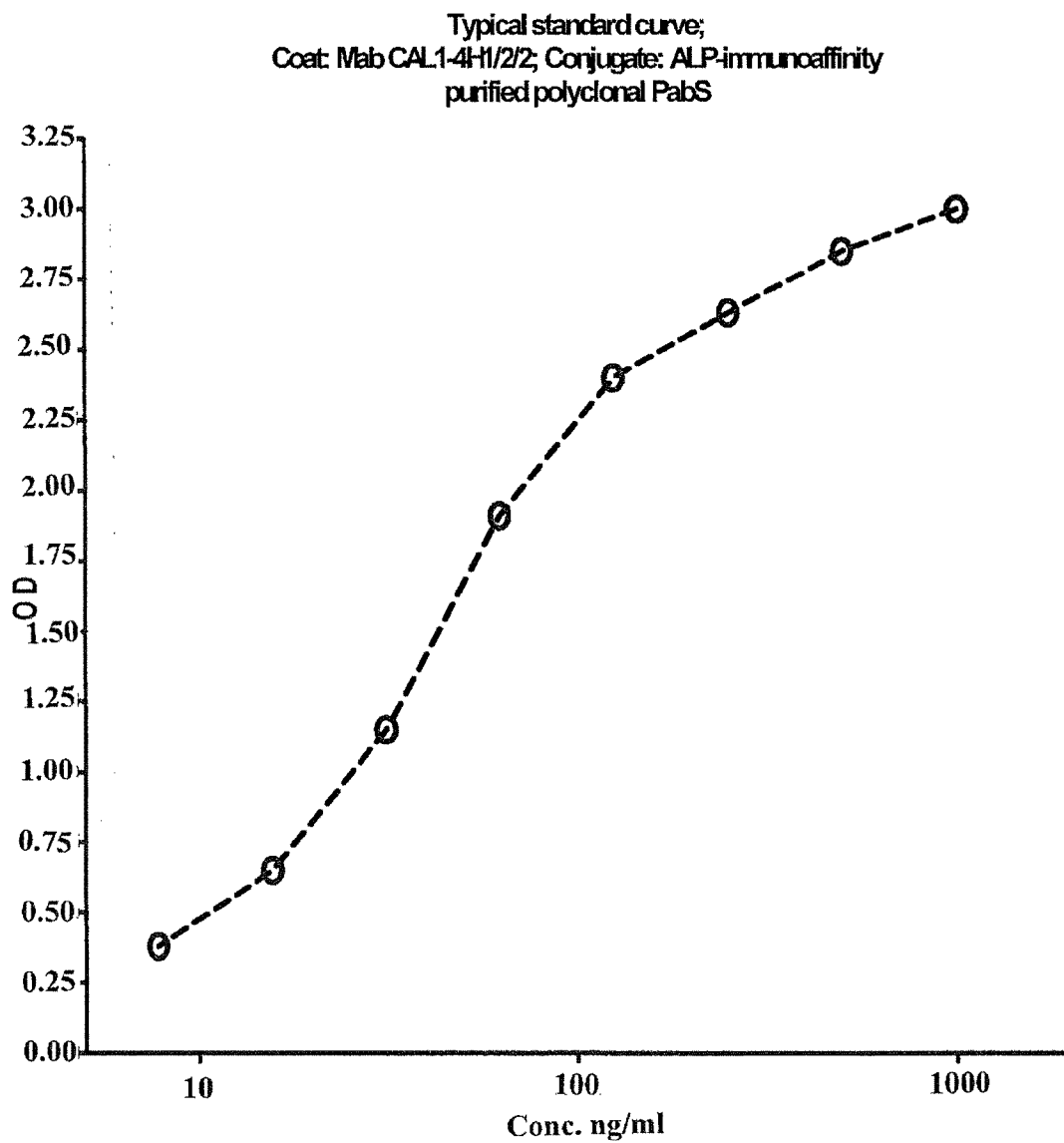


FIGURE 2

Figure 3

Standard curve for an ELISA using
one monoclonal antibody both for coating
of wells and as enzyme conjugate

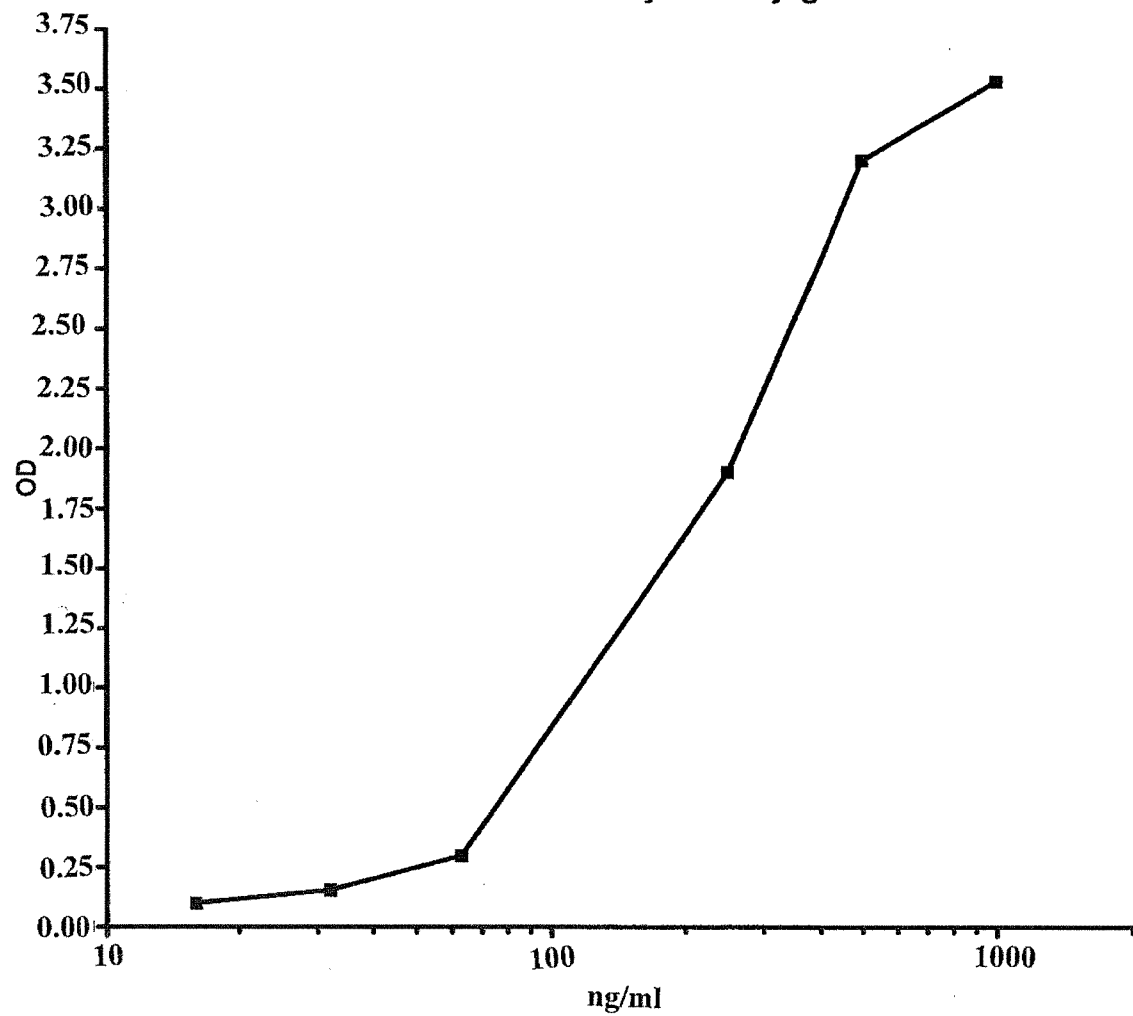


Figure 4
Correlation between estimates of stool extracts
using recombinant calprotectin or S100A9 as standards

