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(54) Title: IMPROVED ELISA IMMUNOASSAY FOR CALPROTECTIN

(57) Abstract: An improved immunoassay for S100A9 provides increased reproducibility and accuracy for the determination of calprotectin, allowing more accurate diagnosis and screening for inflammatory bowel disease. In particular, the immunoassay is a sandwich immunoassay that uses an unlabeled monoclonal anti-S100A9 antibody as the capture antibody and a labeled polyclonal anti-calprotectin antibody as the detection antibody. Further improvements in reproducibility and accuracy are obtained by using citrate buffer as a coating buffer to coat a solid support, such as used in an ELISA assay, with the capture antibody. In general, the method comprises the steps of: (1) providing a monoclonal anti-S100A9 antibody; (2) coating the monoclonal anti-S100A9 antibody onto a solid support; (c) reacting the solid support with coated monoclonal anti-S100A9 antibody with a sample that may contain calprotectin to bind any calprotectin in the sample to the solid support; (d) washing the solid support; (e) reacting the solid support with a labeled polyclonal anti-calprotectin antibody to bind the labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support, the labeled polyclonal antibody being able to produce a detectable signal proportional to the concentration of the antibody bound to the calprotectin bound to the solid support; and (f) measuring the detectable signal to determine the concentration of calprotectin in the sample. The invention further includes diagnostic methods, methods of screening antibodies, and kits.

IMPROVED ELISA IMMUNOASSAY FOR CALPROTECTIN

by

Einar M0rk, Inge Dale, and Magne K. Fagerhol

CROSS-REFERENCES

[0001] This application claims the benefit of United States Provisional Application Serial No. 61/607,131 by E. M0rk et al., filed March 6, 2012, and entitled "Improved ELISA Immunoassay for Calprotectin," the contents of which are incorporated herein in their entirety by this reference.

FIELD OF THE INVENTION

[0002] This invention is directed to an improved immunoassay format for protein or polypeptide analytes, particularly the protein calprotectin.

BACKGROUND OF THE INVENTION

[0003] The protein calprotectin is used as a marker for a number of inflammatory conditions, including, but not limited to, inflammatory bowel disease (Crohn's disease or ulcerative colitis), cystic fibrosis, psoriasis, rheumatoid arthritis, and chronic bronchitis. Calprotectin is also associated with a number of other inflammatory disease processes, such as allograft rejection, coronary heart disease, atheromatosis, sepsis, preeclampsia, cirrhosis, periodontitis, obesity, type 2 diabetes, acne vulgaris, asthma, autoimmune disorders, chronic prostatitis, glomerulonephritis, pelvic inflammatory disease, sarcoidosis, and vasculitis. Additionally, inflammatory processes associated with calprotectin contribute to the etiology of cancer, including pancreatic cancer, ovarian cancer, and uterine cancer, and are especially prominent in inflammatory breast

cancer. Calprotectin is especially useful as a marker for the diagnosis of inflammatory bowel disease, for screening for inflammatory bowel disease, and for determining the prognosis of inflammatory bowel disease in patients already diagnosed with that condition.

[0004] Calprotectin belongs to the S100 family of proteins. The name derives from the fact that they are resistant to precipitation by ammonium sulfate, so that they are soluble even in 100 per cent saturated (thus S100) ammonium sulfate solution. It is believed that they have evolved by a large number of point mutations, 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. Calprotectin is a heterotrimer consisting of two subunits called S100A9 (A9) and one called S100A8 (A8). In the presence of calcium calprotectin will form dimers, while S100A12 (hereafter called A12) will form oligomers, mostly dimers, tetramers, and hexamers. In calprotectin, each of these subunits (the S100A8 subunit and the S100A9 subunit), can bind two calcium ions, for a total of six calcium ions bound per calprotectin molecule.

[0005] Both calprotectin and A12 are abundant in neutrophil granulocytes and monocytes and are released from these cells during inflammation or cell damage or death. They are therefore found in increased concentration in blood, other bodily fluids, secretions, and excretions during inflammation, for which they may be useful markers.

[0006] Inflammatory bowel disease (ulcerative colitis and Crohn's disease) is an extremely severe condition of the digestive tract. Although generally not life-threatening, it is a condition that severely impacts the quality of life and can substantially disrupt a patient's lifestyle, livelihood and relationships. Moreover, patients with inflammatory bowel disease have an increased risk of colorectal cancer, and there is recent research that suggests that patients with inflammatory bowel disease have an increased risk of endothelial dysfunction and coronary artery disease.

[0007] Inflammatory bowel disease is a chronic disease for which there is no certain cure, although the symptoms can be treated by a number of drugs as well as

measures such as dietary changes. Chronic inflammatory bowel disease is characterized by unpredictable flare-ups of symptoms. Such flare-ups of symptoms can severely impair the quality of life of patients with inflammatory bowel disease. Most patients have recurrent periods of active disease, with significant symptoms, or even continuous active inflammation. For patients who do respond to treatment, the duration of the response varies considerably. The course of the disease, the variability of the response to existing treatments, and the unpredictability of flare-ups all make accurate monitoring of the course of the disease a necessity. This is especially significant during periods of low disease activity, when it is important to be able to detect subclinical intestinal inflammation and to be able to predict the possible occurrence of relapses.

[0008] A number of markers have been proposed for use for monitoring inflammatory bowel disease. These markers, in addition to calprotectin, include immunoassays for anti-*Saccharomyces cerevisiae* antibody (ASCA) (either IgG or IgA), anti OmpC *(Escherichia coli* outer membrane porin) antibody, and anti-CBirl (flagellin protein) antibody. There are also additional assays for IBD-specific pANCA (perinuclear antineutrophilic cytoplasmic antibody): autoantibody ELISA, IFA perinuclear staining, and DNase sensitivity. Some of these tests are described in T.D. Jaskowski, "Analysis of Serum Antibodies in Patients Suspected of Having Inflammatory Bowel Disease," Clin. Vacc. Immunol. 13: 655-660 (2006), incorporated herein by this reference. However, these assays have had little acceptance in the medical community. Another alternative immunoassay technique proposed involves diagnosis based on the level of anti-glycan antibodies. Specifically, the antibodies detected are one or more of anti-GIc (β) antibody, anti-GIc (β1-4) Glc (β) antibody, anti-GIc (β1-3) Glc (β) antibody, anti-GlcNAc 6-sulfate antibody, anti-dextran antibody, anti-xylan antibody, anti-GlcNAc (β 1-4) GlcNac (β) antibody, anti-Gal-3-sulfate (β) antibody, anti-GlcNAc (β 1-3) GalNAc (β) antibody, anti-GlcNAc (β 1-3) Gal (β 1-4) Glc (β) antibody, or anti-Gal (a 1-3) Gal (β 1-4) GlcNAc (β) antibody. These assays also have seen little use. Another marker that has been proposed for the diagnosis, detection, or monitoring of inflammatory bowel disease is lactoferrin. However, lactoferrin is not nearly as specific a marker for inflammatory bowel disease as is calprotectin. Additional Additional potential

immunoassays employing fecal biomarkers are described in A.D. Sutherland et al., "Review of Fecal Biomarkers in Inflammatory Bowel Disease," DOI: 10.1 007/s 10350-008-930-8 (2008). The markers considered, in addition to calprotectin and lactoferrin, included lysozyme, polymorphonuclear neutrophil elastase, human neutrophil lipocalin, myeloperoxidase, eosinophil-derived proteins, a_1-antitrypsin, and nitric oxide. These markers were considered not particularly useful or significant for diagnosis.

[0009] Other non-immunological diagnostic and assay methods are known. These methods are described, for example, in M.R. Konikoff & L.A. Denson, "Role of Fecal Calprotectin as a Biomarker of Intestinal Inflammation in Inflammatory Bowel Disease," Inflamm. Bowel Pis. 12: 524-533 (2006); in this publication, endoscopy with biopsies is described as the "gold standard" for assessing intestinal inflammation. This cannot be done routinely because it is far too invasive. This publication also mentions other imaging methods such as CT and MRI exams, barium enemas, and enteroclysis, but these methods also cannot be performed routinely. Also known is the labeling of leukocytes with the radioactive isotopes ^{111}In and ^{99}Tc with subsequent fecal monitoring of these radioactive isotopes. Despite the high sensitivity and specificity of such radioactive techniques, they have substantial limitations; they are cumbersome, costly, difficult to perform, and expose the patient, particularly the liver and spleen, to radiation. Therefore, the use of such radioactive techniques is not recommended for children, adolescents, or fertile women.

[0010] Therefore, the most practical and specific biomarker for the diagnosis, detection, or monitoring of inflammatory bowel disease is calprotectin. Although a number of assays for calprotectin are known, there is a necessity for an improved assay for calprotectin that is specific for calprotectin and has a wide dynamic range, such that it is not susceptible to interference that can occur. Additionally, there is a necessity for an improved assay for calprotectin that is highly specific for calprotectin and can distinguish calprotectin from other closely related S 100 proteins.

SUMMARY OF THE INVENTION

[001 1] Greatly improved specificity and reproducibility in the assay of calprotectin can unexpectedly be achieved by the use of a monoclonal anti-S1 00A9 antibody as unlabeled capture antibody and a labeled polyclonal anti-calprotectin antibody as detection antibody in a sandwich immunoassay performed on a solid support, such as an ELISA. The label of the detection antibody can be an enzyme label such as alkaline phosphatase. Furthermore, additional improvements in specificity and reproducibility can unexpectedly result if citrate buffer is used to coat the solid support with the unlabeled monoclonal anti-S1 00A9 capture antibody used in the sandwich assay, such as an ELISA assay.

[0012] Accordingly, one aspect of the present invention is a method for determining the concentration of calprotectin in a sample comprising the steps of:

(1) providing a monoclonal anti-S100A9 antibody;

(2) coating the monoclonal anti-S100A9 antibody onto a solid support;

(3) reacting the solid support with coated monoclonal anti-S1 00A9 antibody with a sample that may contain calprotectin to bind any calprotectin in the sample to the solid support;

(4) washing the solid support;

(5) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support, the labeled polyclonal anti-calprotectin antibody being able to produce a detectable signal proportional to the concentration of the antibody bound to the calprotectin bound to the solid support; and

(6) measuring the detectable signal to determine the concentration of calprotectin in the sample.

[0013] The monoclonal antibody to S100A9 can be produced using a recombinant S100A9 protein as immunogen. The recombinant S100A9 protein can have the sequence MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVI EHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP (SEQ ID NO: 1). The recombinant S100A9 protein can be produced by expression of a

DNA molecule with the sequence
GGTACCATATGACCTGCAAAATGAGCCAGCTGGAACGTAACATTGAAACCATCATC
AACACCTTTCATCAGTATAGCGTGAAACTGGGCCATCCGGATACCCTGAACCAGG
GCGAATTTATGATCGAACACATCATGGAAGATCTGGATACCAACGCGGATAAACAG
CTGTCTTTCGAAGAAGAACTGGTGCGTAAAGATCTGCAGAACTTCCTGAAAAAAGA
AAACAAAAACGAAAAAGAATTTATTATGCTGATGGCGCGTCTGACCTGGGCGAGCC
ATGAAAAAATGCATGAAGGCGATGAAGGCCCGGGTCATCATCATAAACCGGGCCT
GGGCGAAGGCACCCCGTGATAACTCG (SEQ ID NO: 3) in *Escherichia coli.*

[0014] In one alternative, the labeled polyclonal anti-calprotectin antibody is an enzyme-labeled antibody, and the method comprises the step of incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce the detectable signal. In this alternative, the enzyme can produce a product that is detected and/or quantitated photometrically, or the enzyme can produce a product that is detected and/or quantitated by a technique selected from the group consisting of detection and/or quantitation of fluorescence, detection and/or quantitation of chemiluminescence, and detection and/or quantitation of bioluminescence. The enzyme of the enzyme-labeled antibody can be selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase, although other enzymes can be used. A particularly preferred enzyme is alkaline phosphatase, for which a particularly preferred substrate is p-nitrophenylphosphate.

[0015] Typically, the buffer used to coat the solid support with the monoclonal antibody is a citrate buffer. A particularly preferred citrate buffer is 0.1 M sodium citrate, pH 6.0.

[0016] Typically, the solid support is constructed of a synthetic resin selected from the group consisting of polystyrene, Luran, polypropylene, and polyvinyl chloride. Typically, the monoclonal antibody is used to coat the solid support at a concentration of from about 1 to 4 μg/mL. Typically, during the process of coating the solid support, the solid support is stored at a temperature of from about 0° C to about 8° C; however, a higher temperature, of from about 9° C to about 37° C. Typically, during the process of

coating the solid support, the solid support is stored for an incubation period of from about 6 hours to several weeks. Typically, the size of the sample is from about 50 µl to about 150 µl per assay or standard to be performed.

[0017] Typically, the method further comprises the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve. The standard curve can be constructed using either a plurality of concentrations of purified calprotectin or a plurality of concentrations of purified A9 polypeptide, such as recombinant S100A9 polypeptide.

[0018] Preferably, the labeled polyclonal anti-calprotectin antibody is an enzyme-conjugated highly immunoaffinity purified polyclonal anti-calprotectin antibody.

[0019] Typically, in the performance of this assay, the sample is selected from the group consisting of a gastrointestinal tract sample and a fecal sample.

[0020] Typically, the sample is extracted prior to performance of the assay by a method comprising the steps of:

(a) mixing a small amount of sample with an excess amount of aqueous extraction buffer comprising at least one dissociating, disaggregating, and/or chelating agent;

(b) homogenizing the sample in a closed tube; and

(c) recovering the liquid extract resulting from the separation, which contains calprotectin as well as other proteins.

[0021] In this extraction method, typically, a suitable buffer is a Tris buffer with a pH of about 8 with added citrate. The dissociating, disaggregating, and/or chelating agent can be selected from the group consisting of sodium dodecyl sulfate and urea.

[0022] Another aspect of the present invention is a method for diagnosing or detecting the presence of active inflammatory bowel disease comprising the steps of:

(1) providing a monoclonal anti-S100A9 antibody;

(2) coating the monoclonal anti-S100A9 antibody onto a solid support;

(3) reacting the solid support with coated monoclonal anti-S100A9 antibody with a stool sample from a patient suspected of having inflammatory bowel disease to bind any calprotectin in the sample to the solid support;

(4) washing the solid support;

(5) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support;

(6) incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody to produce a detectable signal;

(7) measuring the detectable signal to determine the concentration of calprotectin in the sample; and

(8) determining the presence or absence of active inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case active inflammatory bowel disease is present in the patient, or is less than 50 mg/kg, in which case active inflammatory bowel disease is absent in the patient.

[0023] Typically, in this method, the immunoassay for calprotectin is performed as described above.

[0024] Yet another aspect of the present invention is a method for determining whether a patient with inflammatory bowel disease who has been in remission is at risk of suffering a relapse comprising the steps of:

(1) providing a monoclonal anti-S100A9 antibody;

(2) coating the monoclonal anti-S100A9 antibody onto a solid support;

(3) reacting the solid support with coated monoclonal anti-S1 00A9 antibody with a stool sample from a patient with inflammatory bowel disease that is in remission to bind any calprotectin in the sample to the solid support;

(4) washing the solid support;

(5) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support;

(6) incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody to produce a detectable signal;

(7) measuring the detectable signal to determine the concentration of calprotectin in the sample; and

(8) determining the risk of relapse of inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease exists, or is less than 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease does not exist.

[0025] Typically, in this method, the immunoassay for calprotectin is performed as described above.

[0026] Another aspect of the invention is a method of selecting a polyclonal or monoclonal anti-S100A9 antibody for high binding affinity to S100A9 polypeptide comprising the steps of:

(1) providing a plurality of polyclonal or monoclonal antibodies binding S100A9 protein;

(2) separately reacting each of the polyclonal or monoclonal antibodies with recombinant S100A9 protein under conditions where equilibrium is reached;

(3) determining the affinity of each of the polyclonal or monoclonal antibodies for S100A9 polypeptide; and

(4) selecting the polyclonal or monoclonal antibody with the highest affinity for S100A9 polypeptide.

[0027] In this method, the antibodies provided for selection can be either polyclonal antibodies or monoclonal antibodies. The method can further comprise the step of comparing the affinity of the antibody selected in step (4) with a standard antibody. The standard antibody can be Calpro Mab CAL1-4H1/2/2. Typically, the antibody selected in step (4) has an affinity of at least 80% of the affinity of Calpro Mab CAL1-4H1/2/2. Preferably, the antibody selected in step (4) has an affinity of at least 90% of the affinity of Calpro Mab CAL1-4H1/2/2. More preferably, the antibody selected in step (4) has an affinity of at least 95% of the affinity of Calpro Mab CAL1-4H1/2/2. Most preferably, the antibody selected in step (4) has an affinity of at least 97.5% of the affinity of Calpro Mab CAL1-4H1/2/2.

[0028] Another aspect of the invention is a method of selecting a polyclonal or monoclonal anti-S100A9 antibody for low cross-reactivity to a polypeptide selected from the group consisting of S100A8 polypeptide and S100A12 polypeptide comprising the steps of:

(1) providing a polyclonal or monoclonal antibody binding S100A9 protein;

(2) reacting the polyclonal or monoclonal antibody with recombinant S100A9 protein under conditions where equilibrium is reached; and

(3) determining the affinity of the polyclonal or monoclonal antibody for S100A9 protein;

(4) reacting the polyclonal or monoclonal antibody with a recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein under conditions where equilibrium is reached;

(5) determining the affinity of the polyclonal or monoclonal antibody for the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein;

(6) comparing the affinity of the polyclonal or monoclonal antibody for recombinant S100A9 protein and for the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein to determine the cross-reactivity of the polyclonal or monoclonal antibody with the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein; and

(7) selecting the polyclonal or monoclonal antibody with the lowest cross-reactivity with the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein.

[0029] In this method, the antibodies provided for selection can be either polyclonal antibodies or monoclonal antibodies. The antigen used to test cross-reactivity can be either S100A8 protein or S100A12 protein. Typically, the antibody selected in step (7) has a cross-reactivity with the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein of

no greater than about 0.1 0. Preferably, the antibody selected in step (7) has a cross-reactivity with the recombinant protein selected from the group consisting of recombinant S 100A8 protein and recombinant S 100A1 2 protein of no greater than about 0.05. More preferably, the antibody selected in step (7) has a cross-reactivity with the recombinant protein selected from the group consisting of recombinant S 100A8 protein and recombinant S100A1 2 protein of no greater than about 0.02.

[0030] Another aspect of the present invention is a method of immunoaffinity purification of polyclonal anti-calprotectin antibodies to remove antibodies against epitopes present on standards but not on calprotectin in actual stool samples comprising the steps of:

(1) providing a polyclonal anti-S100A9 antibody;

(2) performing immunoaffinity purification with an affinity ligand selected from the group consisting of a recombinant calprotectin subunit and a related S 100 protein so that antibody molecules binding the affinity ligand are retained by the affinity ligand; and

(3) isolating a fraction of polyclonal antibody not retained by the affinity ligand in order to remove antibodies against epitopes present on standards but not on calprotectin in actual stool samples.

[0031] In this method, the affinity ligand can be the S 100A8 subunit or the S 100A1 2 subunit. In another alternative, the affinity ligand can be a S 100 protein selected from the group consisting of S 100A1 protein, the S 100A2 protein, the S 100A3 protein, the S 100A4 protein, the S100A5 protein, the S 100A6 protein, the S 100A7 protein, the S 100A1 0 protein, the S 100A1 1 protein, the S 100A1 2 protein, the S 100A13 protein, the S 100A14 protein, the S 100A1 5 protein, and the S 100A1 6 protein.

[0032] Yet another aspect of the present invention is a method for the selection and removal of anti-S1 00A9 antibodies cross-reacting with another S 100 protein other than S100A8 or S 100A9 comprising the steps of:

(1) providing a polyclonal anti-S100A9 antibody;

(2) performing immunoaffinity purification with at least one affinity ligand that is a S 100 protein other than S 100A8 or S 100A9; and

(3) isolating a fraction of polyclonal antibody not retained by the affinity ligand in order to remove anti-calprotectin antibodies that cross-react with at least one S100 protein other than S100A8 or S100A9.

[0033] In this method, the step of performing affinity purification can be performed employing multiple ligands in a single affinity column. The multiple ligands can include include at least two ligands selected from the group consisting of the S100A1 protein, the S100A2 protein, the S100A3 protein, the S100A4 protein, the S100A5 protein, the S100A6 protein, the S100A7 protein, the S100A10 protein, the S100A11 protein, the S100A12 protein, the S100A13 protein, the S100A14 protein, the S100A15 protein, and the S100A16 protein. In another alternative, the step of performing affinity purification is performed employing multiple ligands wherein each ligand is bound to an individual affinity column, and multiple affinity columns are employed. The multiple ligands can include at least two ligands selected from the group consisting of the S100A1 protein, the S100A2 protein, the S100A3 protein, the S100A4 protein, the S100A5 protein, the S100A6 protein, the S100A7 protein, the S100A10 protein, the S100A11 protein, the S100A12 protein, the S100A13 protein, the S100A14 protein, the S100A15 protein, and the S100A16 protein.

[0034] Yet another aspect of the present invention is a method for the selection of anti-S100A9 antibody preparations that give correct readings and provide a wide assay range in an ELISA comprising the steps of:

(1) providing an unlabeled monoclonal anti-S100A9 antibody and an enzyme-labeled polyclonal anti-calprotectin antibody that have been shown to give accurate results in an ELISA assay with stool samples;

(2) providing a candidate preparation of unlabeled monoclonal anti-Si 00A9 antibody;

(3) providing a candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody;

(4) performing an ELISA sandwich immunoassay using the unlabeled monoclonal anti-S100A9 antibody and the enzyme-labeled polyclonal anti-calprotec antibody of (1) using a plurality of stool samples;

(5) performing an ELISA sandwich immunoassay using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody;

(6) comparing the results from the ELISA sandwich immunoassays of steps (4) and (5) to determine whether the results are substantially equivalent; and

(7) selecting candidate preparations of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that produce substantially equivalent results on the plurality of stool samples, such that results obtained using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody differ from the results obtained using a preparation of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that have been shown to give accurate results on stool samples vary by no more than about 10% for any stool sample tested.

[0035] Typically, in this method, the results obtained using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody differ from the results obtained using a preparation of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that have been shown to give accurate results on stool samples vary by no more than about 5% for any stool sample tested.

[0036] Yet another aspect of the present invention is a method of performing an immunoassay for S100A9 and for a related antigen to determine whether inflammatory bowel disease exists in a subject, the method comprising the steps of:

(1) providing a stool sample from a subject;

(2) performing an ELISA sandwich immunoassay for S100A9 using an unlabeled monoclonal anti-S100A9 antibody and an enzyme-labeled polyclonal anti-calprotectin antibody;

(3) performing an immunoassay for a related antigen;

(4) determining the concentration of calprotectin in the stool sample from the results of the ELISA sandwich immunoassay for S100A9;

(5) determining the concentration of the related antigen in the stool sample from the results of the immunoassay for the related antigen; and

(6) comparing the concentration of S100A9 polypeptide and the concentration of the related antigen in the stool sample with the cutoff values for S100A9 polypeptide and the related antigen to determine whether or not inflammatory bowel disease exists in the subject.

[0037] In this method, the related antigen can be S100A12 or lactoferrin.

[0038] Yet another aspect of the present invention is a kit for immunoassay of S100A9 and diagnosis, screening, or monitoring of inflammatory bowel disease comprising, separately packaged:

(1) a quantity of an unlabeled monoclonal anti-S100A9 antibody suitable for performance of an ELISA sandwich immunoassay for the detection of S100A9 polypeptide; and

(2) a quantity of an enzyme-labeled polyclonal anti-calprotectin antibody suitable for performance of an ELISA sandwich immunoassay for the detection of S100A9 polypeptide.

[0039] In one alternative, the kit can include a quantity of an unlabeled monoclonal anti-S100A9 antibody and a quantity of a labeled polyclonal anti-calprotectin antibody each sufficient for a single assay. In another alternative, the kit can include a quantity of an unlabeled monoclonal anti-S100A9 antibody and a quantity of a labeled polyclonal anti-calprotectin antibody sufficient for a plurality of assays.

[0040] The kit can further include, separately packaged, at least one of:

(3) a solid support suitable for performance of the ELISA immunoassay;

(4) wash buffer for washing the solid support after coating the solid support with immobilized antibody and after applying the sample to the solid support;

(5) substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody; and

(6) a set of S100A9 standards of differing concentrations so that a standard curve for S100A9 can be established.

[0041] Typically, in the kit, the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase. Preferably, the enzyme is alkaline phosphatase. When the enzyme is alkaline phosphatase, the kit typically includes, packaged separately, p-nitrophenylphosphate as a substrate for the alkaline phosphatase.

[0042] Another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 polypeptide comprising:

(1) a solid support for lateral flow;

(2) a labeled anti-calprotectin antibody that is either present on the device before the commencement of the immunoassay or is added to the device at the commencement of the immunoassay; and

(3) a detection zone on the solid support comprising immobilized anti-Si 00A9 antibody such that any S100A9 polypeptide in the sample forms an antibody-antigen-antibody sandwich with labeled anti-calprotectin antibody and with the immobilized anti-S100A9 antibody in the detection zone.

[0043] In the device for performing a lateral flow sandwich immunoassay, typically, the solid support is constructed of a material selected from the group consisting of nitrocellulose, glass fiber, paper, nylon, and a synthetic nanoporous polymer. Preferably, the solid support is nitrocellulose.

[0044] In one alternative, the labeled anti-calprotectin antibody is present on the device before the commencement of the immunoassay. In another alternative, the labeled anti-calprotectin antibody is added to the device at the commencement of the immunoassay.

[0045] The device can further comprise a control zone. In one alternative, the control zone comprises an immobilized antibody capable of specifically binding the labeled anti-calprotectin antibody by binding to a portion of the antibody distinct from the paratope of the labeled anti-calprotectin antibody. In another alternative, the control

zone comprises an immobilized unrelated antigen and the device comprises a labeled antibody specifically binding the immobilized unrelated antigen.

[0046] The device can also further comprise a casing having at least one aperture therein.

[0047] In one alternative, the device comprises:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(3) a conjugate zone having mobilizable labeled anti-calprotectin antibody located on the solid support; and

(4) a sample pad for application of a sample in operable contact with the first end of the solid support;

wherein a sample applied to the sample pad migrates to the conjugate zone to solubilize and mobilize the labeled anti-calprotectin antibody and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0048] In another alternative, the device comprises:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support; and

(3) a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the first end of the solid support;

wherein a sample applied to the conjugate pad solubilizes and mobilizes the labeled anti-calprotectin antibody in the conjugate pad and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the

immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0049] In yet another alternative, the device can comprise

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(3) a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the first end of the solid support; and

(4) a sample pad for application of the sample in operable contact with the conjugate pad;

wherein a sample applied to the sample pad migrates to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0050] In still another alternative, the device can comprise:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a conjugate pad having mobilizable labeled anti-calprotectin antibody; and

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the

sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0051] In yet another alternative, the device can comprise:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the sample pad; and

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the sample pad and the sample migrates to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0052] In still another alternative, the device can comprise:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(3) a sample pad in operable contact with the first end of the solid support;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the sample pad, the sample and the mobile labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the

labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0053] In yet another alternative, the device can comprise:

(1) a solid support providing lateral flow having a first end and a second end; and

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the solid support, the sample and the mobile labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0054] In still another alternative, the device can comprise:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a conjugate pad for application of a mobile labeled anti-calprotectin antibody in operable contact with the sample pad; and

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the sample pad and a mobile labeled anti-calprotectin antibody is applied to the conjugate pad, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the sample pad and conjugate pad are placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0055] In yet another alternative, the device can comprise:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a mobile labeled anti-calprotectin antibody to the sample pad;

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the sample pad, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the sample pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0056] Typically, the label of the labeled anti-calprotectin antibody is a directly detectable label. The directly detectable label can be selected from the group consisting of a radioactive label, a fluorescent label, a chemiluminescent label, an electrochemiluminescent label, a bioluminescent label, a colloidal metal label, a colloidal metal oxide label, a dye label, a colored latex particle label, a colored polystyrene or polypropylene particle label, a liposome label, a Surface-Enhanced Resonant Raman Spectroscopy (SERRS) label, and a signaling aptamer label. A particularly preferred label is a colloidal metal label, such as a colloidal gold or silver label. A more particularly preferred label is a colloidal gold label.

[0057] The immobilized anti-S100A9 antibody can be covalently or non-covalently immobilized at the detection zone. The labeled anti-calprotectin antibody can be a polyclonal or monoclonal antibody.

[0058] Lateral flow devices according to the present invention can be employed in immunoassays as described above. For example, a method for determining the concentration of calprotectin in a sample comprises the steps of:

(1) applying the sample to a lateral flow device according to the present invention as described above;

(2) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody; and

(3) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone.

[0059] The method can further comprise the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

[0060] Similarly, a method of detecting or diagnosing the presence of active inflammatory bowel disease comprises the steps of:

(1) applying a sample that may contain calprotectin to a lateral flow device according to the present invention as described above;

(2) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(3) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone; and

(4) determining the presence or absence of active inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case active inflammatory bowel disease is present in the patient, or is less than 50 mg/kg, in which case active inflammatory bowel disease is absent in the patient.

[0061] Also similarly, a method of determining whether a patient with inflammatory bowel disease who has been in remission is at risk of suffering a relapse comprises the steps of:

(1) applying a sample that may contain calprotectin to a lateral flow device according to the present invention as described above;

(2) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(3) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone; and

(4) determining the risk of relapse of inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease exists, or is less than 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease does not exist.

[0062] Also, similarly, a method of performing an immunoassay for S100A9 polypeptide and for a related antigen to determine whether inflammatory bowel disease exists in a subject comprises the steps of:

(1) providing a stool sample from a subject;

(2) applying the sample to a lateral flow device according to the present invention as described above;

(3) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(4) performing an immunoassay for a related antigen;

(5) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone;

(6) determining the concentration of the related antigen in the stool sample from the results of the immunoassay for the related antigen; and

(7) comparing the concentration of S100A9 polypeptide and the concentration of the related antigen in the stool sample with the cutoff values for S100A9 polypeptide and the related antigen to determine whether or not inflammatory bowel disease exists in the subject.

[0063] In this method, the related antigen can be S100A12 or lactoferrin.

[0064] Yet another aspect of the invention is a kit for immunoassay of S100A9 and diagnosis, screening, or monitoring of inflammatory bowel disease comprising, separately packaged:

(1) a solid support for performing a lateral flow sandwich immunoassay for detection of S100A9 polypeptide; and

(2) a labeled anti-calprotectin antibody to be applied to the solid support.

[0065] In one alternative, the kit can include a single solid support and a quantity of labeled anti-calprotectin antibody sufficient for a single assay. In another alternative, the kit can include a plurality of solid supports and a quantity of labeled anti-calprotectin antibody sufficient for a plurality of assays such that the quantity of labeled anti-calprotectin antibody is sufficient for use with each of the solid supports included in the kit.

[0066] The label of the labeled anti-calprotectin antibody can be an enzyme label, in which case the kit can further comprise, separately packaged, a substrate for the enzyme label. Additionally, the kit can further comprise, separately packaged, a set of S100A9 polypeptide standards of differing concentrations so that a standard curve for S100A9 polypeptide can be established.

BRIEF DESCRIPTION OF THE DRAWINGS]

[0067] These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description, appended claims, and accompanying drawings where:

[0068] Figure 1A depicts a top view of a first alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is present in the device before the commencement of the assay.

[0069] Figure 1B depicts a side view of the first alternative for an assay device that can perform a sandwich immunoassay of Figure 1A showing an optional backing and casing.

[0070] Figure 2A depicts a top view of a second alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is present in the device before the commencement of the assay.

[0071] Figure 2B depicts a side view of the second alternative for an assay device that can perform a sandwich immunoassay of Figure 2A showing an optional backing and casing.

[0072] Figure 3A depicts a third alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is present in the device before the commencement of the assay.

[0073] Figure 3B depicts a side view of the third alternative for an assay device that can perform a sandwich immunoassay of Figure 3A showing an optional backing and casing.

[0074] Figure 4 depicts a fourth alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is present in the device before the commencement of the assay.

[0075] Figure 5 depicts a fifth alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is present in the device before the commencement of the assay.

[0076] Figure 6A depicts a first alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is added to the device at the commencement of the assay.

[0077] Figure 6B depicts a side view of the first alternative for an assay device that can perform a sandwich immunoassay of Figure 6A showing an optional backing and casing.

[0078] Figure 7A depicts a second alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is added to the device at the commencement of the assay.

[0079] Figure 7B depicts a side view of the second alternative for an assay device that can perform a sandwich immunoassay of Figure 7A showing an optional backing and casing.

[0080] Figure 8 depicts a third alternative for an assay device that can perform a sandwich immunoassay in which the labeled anti-calprotectin antibody is added to the device at the commencement of the assay.

[0081] Figure 9 is a standard curve for an S100A8 ELISA using monoclonal antibodies.

[0082] Figure 10 is a standard curve for an S100A9 ELISA using unlabeled monoclonal anti-S100A9 antibody as the capture antibody and alkaline-phosphatase-labeled polyclonal anti-S100A9 antibody as the detection antibody, employing purified calprotectin protein as the standard.

[0083] Figure 11 is a standard curve for an S100A9 ELISA using unlabeled monoclonal anti-S100A9 antibody as the capture antibody and alkaline-phosphatase-labeled monoclonal anti-S100A9 antibody as the detection antibody, again employing purified calprotectin protein as the standard.

[0084] Figure 12 is a graph showing the correlation between a standard curve for an S100A9 ELISA using unlabeled monoclonal anti-S100A9 antibody as the capture antibody and alkaline-phosphatase-labeled polyclonal anti-S100A9 antibody as the detection antibody, employing recombinant calprotectin as the standard, and a standard curve for an S100A9 ELISA using unlabeled monoclonal anti-S100A9 antibody as the

capture antibody and alkaline-phosphatase-labeled polyclonal anti-S100A9 antibody as the detection antibody, employing purified recombinant S100A9 as the standard.

DETAILED DESCRIPTION OF THE INVENTION

[0085] An improved immunoassay in ELISA format for calprotectin meets these needs and provides a more accurate and specific immunoassay for detection of calprotectin that can be used to detect, diagnose, or monitor inflammatory bowel disease.

[0086] Both calprotectin and S100A12 are abundant in neutrophil granulocytes and monocytes and are released from these cells during inflammation or cell damage/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 common that are common for the two proteins. Out of thirteen monoclonal antibodies that we have 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.

[0088] 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.

[0088] The original calprotectin ELISA was developed by Fagerhol at Ullevaal University Hospital, Oslo, Norway (here abbreviated as UUH). Antibodies were raised by immunization of rabbits with native calprotectin purified from extracts of neutrophil granulocytes. IgG fractions were isolated from mixtures of sera from 10 rabbits by use of ammonium sulfate precipitation and DEAE ion exchange chromatography. Subsequently, anti-calprotectin antibody was purified by imnnunoaffinity chromatography

using a column with 30 mg calprotectin covalently bound to sepharose beads. A subsequent set of immunizations with rabbits from another source produced antibodies (here abbreviated as PAbS) giving very good results in the sense that the shape of standard curves and the assay range were much better giving an upper limit eight times the original. However, as detailed below, these results were applicable to standard curves using purified antigens, not to stool samples. Because, in the clinical context, assays for calprotectin are performed on stool samples, there is a need to ensure that such an ELISA assay is reliable as applied to stool samples and not merely to purified antigens as used to establish a standard curve.

[0089] However, when stool extracts were tested on the original and new ELISA (i.e., the ELISA using the first and second batches of polyclonal antibodies), the latter yielded results of only 25 to 50 per cent of the former in terms of calprotectin concentration. This led to the idea that the number and structure of epitopes on calprotectin in the standard (i.e., the purified antigens) and in the extracts might differ significantly, and that some antisera might contain antibodies reacting better with the former. The reasoning was partly based upon prior results from running stool extracts on gel permeation chromatography (GPC) showing that calprotectin eluted in high molecular weight (MW) fractions corresponding to 100 to 1000 kDa or larger. 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 more sophisticated extraction buffer was developed, the yield increased to about 50%; when such extracts were run on 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 an altered molecular configuration of calprotectin caused by factors in the gut content. The latter would be more likely to occur in conformational epitopes as opposed to linear epitopes. It is well known in immunochemistry that epitopes occurring on proteins or polypeptides can be divided into linear or sequence epitopes and conformational epitopes. Linear or

sequence epitopes are formed by the primary sequence of the protein or polypeptide and involve amino acid residues that are located adjacent to each other in the primary sequence, while conformational epitopes are formed by the secondary or tertiary sequence of the protein or polypeptide and involve amino acid residues that are not necessarily adjacent to other in the primary sequence, but are brought into proximity by the secondary or tertiary structure of the protein or polypeptide. An important 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.

[0090] To test the hypotheses mentioned above, recombinant calprotectin subunits were prepared, i.e. S100A8 (here abbreviated as A8) and S100A9 (here abbreviated as A9) which spontaneously form heterodimers (here called recombinant calprotectin) when the subunits are incubated with low concentrations of calcium chloride, typically 2 mM. These proteins, as well as recombinant S100A12, a closely related protein, were coated in microwells which allowed testing of different antisera and purified antibodies to determine their reaction spectra. Furthermore, immunoaffinity columns were prepared by coupling A8, A9 and recombinant calprotectin in addition to the native calprotectin column, so that binding and non-binding antibodies could be tested.

[0091] It was found that that the original antibody, from UUH, reacted only against calprotectin, recombinant calprotectin and A9. In contrast, the second preparation of antibody reacted also against A8. Part of that reactivity remained even if the antibodies were passed through an A8 affinity column suggesting that some antibodies reacted with epitopes that are common to A9 and A8. If epitopes on A8 are less available on calprotectin in stools than in the standard, and anti-A8 contributes to signal in the ELISA, falsely low concentrations in samples must be expected. To test this hypothesis, we established a specific A8 ELISA using monoclonal antibodies giving the standard curve shown in Figure 9. The results in Figure 9 represent a standard

curve using purified antigen with an S100A8 ELISA and using anti-S100A8 monoclonal antibody.

[0092] Thirteen stool extracts with calprotectin concentration between 21 and 817 ng/ml were tested on this ELISA, but they all gave a signal clearly below that of the lower standard. This supports the conclusion that antibodies reacting with S100A8 should be avoided in assays for calprotectin in stool extracts.

[0093] We therefore hypothesized that this problem could be solved by the use of only S100A9 as standard. This is in agreement with the principle that for 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 polypeptide.

[0094] 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 extracts started to appear when antisera contain S100A8 epitopes were tried, I chose to start working with mAbs reacting with S100A9 only.

[0095] Another important factor for the net ELISA result is the antibody used for preparation of antibody-enzyme conjugates. The ideal combination of coating antibody and conjugate antibody should give the correct result in stool extract when compared with the original UUH method. We therefore tried a large number of different combinations of mAbs and affinity purified polyclonals for coating or enzyme conjugation.

[0096] To our surprise, excellent results were obtained when one particular mAb was used for coating inadvertently in citrate buffer rather than the standard carbonate buffer, together with an alkaline phosphatase imnnunoaffinity purified polyclonal antibody that had been affinity purified on a native calprotectin column; the polyclonal antibody was the conjugate antibody. For the first time we found similar values in stool samples as obtained with the original ELISA.

[0097] The assays of the present invention are sandwich immunoassays. Sandwich immunoassays are well known in the art. In a sandwich immunoassay, the a

molecule of an analyte to be assayed is bound to two antibody molecules: an immobilized unlabeled antibody located on a solid phase or in a detection zone, and a labeled mobile antibody. Detection of the analyte requires the binding of both antibody molecules to the analyte. As detailed below, sandwich assays for S100A9 polypeptide employing suitable antibody combinations can be performed on either a solid phase platform, basically analogous to a conventional ELISA assay, in which the reagents required are successively added to a solid phase without migration of macromolecular components participating in the sandwich immunoassay reaction, or a lateral flow platform, in which a sample and a labeled anti-calprotectin antibody, and possibly other components, migrate through a solid support for lateral flow with a detection zone including an immobilized anti-S100A9 antibody. Both the solid phase platform and the lateral flow platform are described below.

[0098] Accordingly, one aspect of the present invention is a method for determining the concentration of calprotectin in a sample comprising the steps of:

(1) providing a monoclonal anti-S100A9 antibody;

(2) coating the monoclonal anti-S100A9 antibody onto a solid support;

(3) reacting the solid support with coated monoclonal anti-S100A9 antibody with a sample that may contain calprotectin to bind any calprotectin in the sample to the solid support;

(4) washing the solid support;

(5) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support, the labeled polyclonal anti-calprotectin antibody being able to produce a detectable signal proportional to the concentration of the antibody bound to the calprotectin bound to the solid support; and

(6) measuring the detectable signal to determine the concentration of calprotectin in the sample.

[0099] In one alternative, the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A8. In another alternative, the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an

epitope existing on S100A9. In yet another alternative, the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding a conformational epitope incorporating both S100A8 and S100A9. As used herein, the term "labeled polyclonal anti-calprotectin antibody" refers to any of these three alternatives: (i) an antibody specifically binding an epitope existing on S100A8; (ii) an antibody specifically binding an epitope existing on S100A9; or (iii) an antibody specifically binding a conformational epitope incorporating both S100A8 and S100A9 unless further limited. Additionally, the following labeled polyclonal antibodies are suitable for use in assay devices and methods according to the present invention: (i) a labeled polyclonal antibody that reacts strongly with all of calprotectin, isolated S100A8, and isolated S100A9; (ii) a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A8 but not at all or to a significantly lesser degree with isolated S100A9; and (iii) a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A9 but not at all or to a significantly lesser degree with isolated S100A8. Typically, the labeled polyclonal anti-calprotectin antibody is an enzyme-labeled antibody, and the assay method comprises the step of incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce the detectable signal.

[0100] Typically, the monoclonal antibody to S100A9 is produced using a recombinant S100A9 protein as immunogen. A suitable recombinant S100A9 protein has the sequence

MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVI
EHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP

(SEQ ID NO: 1). The genomic DNA sequence encoding S100A9 is

AAACACTCTGTGTGGCTCCTCGGCTTTGACAGAGTGCAAGACGATGACTTGCAAAA
TGTCGCAGCTGGAACGCAACATAGAGACCATCATCAACACCTTCCACCAATACTCT
GTGAAGCTGGGGCACCCAGACACCCTGAACCAGGGGGAATTCAAAGAGCTGGTG
CGAAAAGATCTGCAAAATTTTCTCAAGAAGGAGAATAAGAATGAAAAGGTCATAGA
ACACATCATGGAGGACCTGGACACAAATGCAGACAAGCAGCTGAGCTTCGAGGAG
TTCATCATGCTGATGGCGAGGCTAACCTGGGCCTCCCACGAGAAGATGCACGAGG
GTGACGAGGGCCCTGGCCACCACCATAAGCCAGGCCTCGGGGAGGGCACCCCCT

AAGACCACAGTGGCCAAGATCACAGTGGCCACGGCCACGGCCACAGTCATGGTG
GCCACGGCCACAGCCACTAATCAGGAGGCCAGGCCACCCTGCCTCTACCCAACC
AGGGCCCCGGGGCCTGTTATGTCAAACTGTCTTGGCTGTGGGGCTAGGGGCTGG
GGCCAAATAAAGTCTCTTCCTCCAAGTCAAAAAAAAAA (SEQ ID NO: 2). The sequence of SEQ ID NO: 2 is derived from mRNA and therefore excludes introns possibly present in the genomic sequence. A DNA sequence encoding A9 that is suitable for expression in *Escherichia coli* is
GGTACCATATGACCTGCAAAATGAGCCAGCTGGAACGTAACATTGAAACCATCATC
AACACCTTTCATCAGTATAGCGTGAAACTGGGCCATCCGGATACCCTGAACCAGG
GCGAATTTATGATCGAACACATCATGGAAGATCTGGATACCAACGCGGATAAACAG
CTGTCTTTCGAAGAAGAACTGGTGCGTAAAGATCTGCAGAACTTCCTGAAAAAAGA
AAACAAAAACGAAAAAGAATTTATTATGCTGATGGCGCGTCTGACCTGGGCGAGCC
ATGAAAAAATGCATGAAGGCGATGAAGGCCCGGGTCATCATCATAAACCGGGCCT
GGGCGAAGGCACCCCGTGATAACTCG (SEQ ID NO: 3); this sequence is optimized for expression in *E. coli.*

[0101] The recombinant S100A9 protein of SEQ ID NO: 1 that is suitable for use as an immunogen can be produced by expression of a DNA sequence encoding SEQ ID NO: 1. One such DNA sequence is SEQ ID NO: 3, which is optimized for expression in *Escherichia coli.*

[0102] SEQ ID NO: 3 is only one example of a DNA sequence that can be used to express the amino acid sequence of SEQ ID NO: 1. Other DNA sequences can readily be constructed by one of ordinary skill in the art employing the known degeneracy of DNA codons in the genetic code. In general, any DNA sequence encoding the amino acid sequence of SEQ ID NO: 1 can be used, although, in some cases, particular DNA sequences are preferred because of codon utilization biases in some organisms used for expression, such as *Escherichia coli* or because of the presence of negative c/s-acting sites such as internal RBS sites, TATA boxes, or Chi recombination sites, which may negatively influence expression. The DNA sequence of SEQ ID NO: 3 is optimized for expression in *E. coli,* with a CAI (codon adaptation index) of 0.97, which indicates that the sequence is extremely well adapted for expression in E.

coli. Other DNA sequences can be selected by one of ordinary skill in the art for expression in other organisms as described below.

[0103] Expression techniques are well known in the art. A variety of host-expression vector systems may be utilized to express the S100A9 polypeptide or other proteins intended to be used as immunogens. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing a suitable coding sequence; yeast transformed with recombinant yeast expression vectors containing the zinc finger-nucleotide binding coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing an appropriate coding sequence; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing an appropriate coding sequence; or animal cell systems infected with recombinant virus expression vectors (e.g., retroviruses, adenovirus, vaccinia virus) containing an appropriate coding sequence, or transformed animal cell systems engineered for stable expression. In such cases where glycosylation may be important, expression systems that provide for translational and post-translational modifications may be used; e.g., mammalian, insect, yeast or plant expression systems.

[0104] Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see e.g., Bitter et al., Methods in Enzymology, 153:516-544, 1987). For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used. When cloning in mammalian cell systems, promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the retrovirus long terminal repeat; the adenovirus late promoter; the vaccinia virus 7.5K promoter) may be used. Promoters produced by recombinant DNA or synthetic

techniques may also be used to provide for transcription of the inserted coding sequence.

[0105] In bacterial systems a number of expression vectors may be advantageously selected. For example, when large quantities are to be produced, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Those which are engineered to contain a cleavage site to aid in recovering the protein are preferred. Such vectors include but are not limited to the *Escherichia coli* expression vector pUR278 (Ruther et al., EMBO J., 2:1 791 ,1983), in which the coding sequence may be ligated into the vector in frame with the lac Z coding region so that a hybrid A9 protein-lac Z protein is produced; pIN vectors (Inouye & Inouye, Nucleic Acids Res. 13:31 01-31 09, 1985; Van Heeke & Schuster, J. Biol. Chem. 264:5503-5509, 1989); and the like.

[0106] In yeast, a number of vectors containing constitutive or inducible promoters may be used. For a review, see Current Protocols in Molecular Biology, Vol. 2, 1988, ed. Ausubel et al., Greene Publish. Assoc. & Wiley Interscience, Ch. 13; Grant, et al., 1987, Expression and Secretion Vectors for Yeast, in Methods in Enzymology, eds. Wu & Grossman, 31987, Acad. Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol. II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987, Heterologous Gene Expression in Yeast, Methods in Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol. 152, pp. 673-684; and The Molecular Biology of the Yeast Saccharomyces, 1982, Eds. Strathern et al., Cold Spring Harbor Press, Vols. I and II. A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (Cloning in Yeast, Ch. 3, R. Rothstein In: DNA Cloning Vol. 11, A Practical Approach, Ed. D M Glover, 1986, IRL Press, Wash., D.C.). Alternatively, vectors may be used which promote integration of foreign DNA sequences into the yeast chromosome.

[0107] In cases where plant expression vectors are used, the expression of a coding sequence may be driven by any of a number of promoters. For example, viral promoters such as the 35S RNA and 19S RNA promoters of CaMV (Brisson et al., Nature, 310:51 1-514, 1984), or the coat protein promoter to TMV (Takamatsu et al.,

EMBO J., 6:307-31 1, 1987) may be used; alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al., EMBO J. 3:1 671 - 1680, 1984; Broglie et al., Science 224:838-843, 1984); or heat shock promoters, e.g., soybean hsp1 7.5-E or hsp17.3-B (Gurley, et al. Mol. Cell. Biol., 6:559-565, 1986) may be used. These constructs can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, microinjection, electroporation, or other techniques known in the art. For reviews of such techniques see, for example, Weissbach & Weissbach, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp. 421 -463, 1988; and Grierson & Corey, Plant Molecular Biology, 2d Ed., Blackie, London, Ch. 7-9, 1988.

[0108] An alternative expression system that can be used to express a protein of the invention is an insect system. In one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The polypeptide coding sequence may be cloned into non-essential regions (in *Spodoptera frugiperda,* for example, the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of the polypeptide coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect cells in which the inserted gene is expressed. (E.g., see Smith et al., J. Biol. 46:584, 1983; Smith, U.S. Pat. No. 4,215,051).

[0109] Eukaryotic systems, and preferably mammalian expression systems, allow for proper post-translational modifications of expressed mammalian proteins to occur. Therefore, eukaryotic cells, such as mammalian cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, phosphorylation, and, advantageously secretion of the gene product are suitable host cells for the expression of a polypeptide such as an S 100A8, S 100A9, or S 100A1 2 polypeptide. Such host cell lines may include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, and WI38. However, as detailed above, expression can also

be obtained in *E. coli* bacterial cells and such expression may be preferable; codon optimization for expression in *E. coli* is described above.

[01 10] Mammalian cell systems that utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the coding sequence of a polypeptide such as an S100A8, S100A9, or S100A12 polypeptide may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene may then be inserted into the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the desired polypeptide in infected hosts (e.g., see Logan & Shenk, Proc. Natl. Acad. Sci. USA 81:3655-3659, 1984). Alternatively, the vaccinia virus 7.5K promoter may be used. (e.g., see, Mackett et al., Proc. Natl. Acad. Sci. USA, 79:7415-7419, 1982; Mackett et al., J. Virol. 49:857-864, 1984; Panicali et al., Proc. Natl. Acad. Sci. USA, 79:4927-4931 , 1982). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver et al., Mol. Cell. Biol. 1:486, 1981). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the desired polypeptide in host cells (Cone & Mulligan, Proc. Natl. Acad. Sci. USA 81:6349-6353, 1984). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionein IIA promoter and heat shock promoters.

[01 11] For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with a cDNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences,

transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in enriched media, and then are switched to a selective medium. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler et al., Cell 11:223, 1977), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, Proc. Natl. Acad. Sci. USA, 48:2026, 1962), and adenine phosphoribosyltransferase (Lowy et al., Cell, 22:81 7, 1980) genes, which can be employed in tk^-, $hgprt^-$ or $aprt^-$ cells respectively. Also, antimetabolite resistance-conferring genes can be used as the basis of selection; for example, the genes for dhfr, which confer resistance to methotrexate (Wigler et al., Natl. Acad. Sci. USA, 77:3567, 1980; O'Hare et al., Proc. Natl. Acad. Sci. USA, 78:1 527, 1981); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, Proc. Natl. Acad. Sci. USA, 78:2072, 1981 ; neo, which confers resistance to the aminoglycoside G41 8 (Colberre-Garapin et al., J. Mol. Biol., 150:1 , 1981); and hygro, which confers resistance to hygromycin (Santerre et al., Gene, 30:147, 1984). Recently, additional selectable genes have been described, namely trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman & Mulligan, Proc. Natl. Acad. Sci. USA, 85:804, 1988); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue L , In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed., 1987).

[01 12] Isolation and purification of microbially expressed protein, or fragments thereof provided by the invention, may be carried out by conventional means including preparative chromatography and immunological separations involving monoclonal or polyclonal antibodies. Antibodies provided in the present invention are immunoreactive with the S100A8 or S100A9 polypeptides; similar antibodies can readily be prepared by techniques well known in the art that are immunoreactive with S 100A1 2 polypeptides if

desired. A particularly suitable anti-S1 00A9 monoclonal antibody is that designated as Calpro Mab CAL1 -4H1/2/2.

[01 13] Once suitably purified preparations of S 100A8 or S 100A9 polypeptides, especially S 100A9 polypeptides, are available, antibodies, including both polyclonal and monoclonal antibodies, can be prepared by standard techniques, such as those disclosed in E. Harlow & D. Lane, "Antibodies: A Laboratory Manual" (Cold Spring Harbor Laboratory, 1988), incorporated herein by this reference. Polyclonal antibodies can be produced by immunization of suitable antibody-producing animals such as rabbits, rats, mice, hamsters, guinea pigs, sheep, or goats with the polypeptides. Typically, immunization is performed with the use of an adjuvant such as Freund's adjuvant, aluminum hydroxide adjuvant, Lipid A, muramyl dipeptide, SAF, or RAS. Once polyclonal antibodies have been prepared and antibody-secreting lymphocytes are available, these cells can be fused with appropriate myeloma fusion partners and hybridomas can be selected that grow in culture and produce monoclonal antibodies of the appropriate specificity according to the protocol originally described by Kohler and Milstein. The steps required for fusion, selection, and screening are well known in the art and are described, for example, in E. Harlow & D. Lane, "Antibodies: A Laboratory Manual" (Cold Spring Harbor Laboratory, 1988), incorporated herein by this reference.

[01 14] The ELISA assay as described herein is a form of sandwich immununoassay. In the ELISA assay, a non-labeled antibody is attached to the surface of a solid phase, such as that of a microtiter well, a magnetic particle, or a plastic bead. This attachment facilitates the separation of bound labeled reactants from free labeled reactants; since only the bound labeled reactants (labeled antibody) bound to the antigen in the sandwich need to be detected or determined.

[01 15] A number of suitable enzymes are known in the art for use in ELISA assays. These enzymes include, but are not limited to, alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase. Other enzyme labels are also known in the art. Such labels include, but are not necessarily limited to, acetate kinase, β-lactamase, glucose oxidase, firefly luciferase, laccase, Renilla luciferase, and xanthine oxidase. Enzyme-labeled antibodies can be

prepared by covalent coupling procedures involving reagents such as, but not limited to, glutaraldehyde, N-succinimidyl 3-[pyridyl] propionate, carbodiimides, carbonyldiimidazole, and other cross-linking reagents known in the art, such as those described in G.T. Hermanson, "Bioconjugate Techniques" (Academic Press, San Diego, 1996), pp. 630-637, incorporated herein by this reference. As another alternative, recombinant antibody conjugates can be prepared by genetic engineering techniques known in the art, the conjugates being produced by transcription and translation of gene fusions.

[01 16] In many cases, the enzyme in the enzyme-labeled antibody in ELISA produces a product that is detected and/or quantitated photometrically, such as by spectroscopy. However, in some alternatives, the enzyme produces a product that is monitored and/or quantitated by other means, such as detection and/or quantitation of fluorescence, bioluminescence, or chemiluminescence. For example, immunoassays that use horseradish peroxidase as the enzyme label can be assayed by the detection of chemiluminescence using a mixture of luminol, peroxide, and an enhancer such as *p*-iodophenol or by using an acridan derivative. Umbelliferone phosphate is a nonfluorescent substrate that is converted to the highly fluorescent umbelliferone by the catalytic activity of the enzyme alkaline phosphatase. Another sensitive assay using an alkaline phosphatase label uses a chemiluminescent adamantyl 1,2-dioxetane aryl phosphate substrate, which is dephosphorylated by the catalytic activity of alkaline phosphate and decomposes with a concomitant long-lived glow of light, such that the detection limit for alkaline phosphatase using this assay can be as low as 1 zeptomole (10^{-21} moles). In another alternative, an enzyme cascade is used. The advantage of the use of an enzyme cascade is that it combines the amplification properties of two enzymes—the alkaline phosphatase present in the antibody label and the second enzyme, alcohol dehydrogenase, in the assay reagent, thereby producing an extremely sensitive assay. For example, the alkaline phosphatase can dephosphorylate NADP to NAD, which then takes part in a reaction catalyzed by alcohol dehydrogenase and the enzyme diaphorase in which ethanol is oxidized to acetaldehyde and *p*-

iodonitrotetrazolium violet is reduced to the detectable formazan by the catalytic activity of diaphorase.

[01 17] Other assay techniques involving enzyme-labeled antibodies are known in the art. For example, enzyme multiplied immunoassay technique (EMIT) is a homogeneous immunoassay. In this technique, antibody against the analyte (S1 00A9 polypeptide) is added together with substrate for an enzyme label to a sample. Binding of the antibody and the antigen (S1 00A9 polypeptide) occurs. An aliquot of a conjugate of an enzyme that can catalyze a reaction involving the substrate and the S 100A9 polypeptide is added to the sample. The enzyme conjugate then binds with any excess antibody to S 100A9 polypeptide, forming an antibody-enzyme-S1 00A9 polypeptide conjugate complex. In the absence of free S 100A9 polypeptide in the original sample, the enzyme is inactivated, such as by the binding of the enzyme in the enzyme-S1 00A9 polypeptide conjugate to the antibody which physically blocks access of the substrate to the active site of the enzyme or by changing the conformation of the enzyme and thus altering the activity. In this assay, which is a homogeneous assay not requiring separation of bound and free label, the greater the concentration of analyte (S1 00A9 polypeptide), the greater is the quantity of enzyme activity.

[01 18] Still another antibody immunoassay format known in the art is cloned enzyme donor immunoassay (CEDIA). In CEDIA, inactive fragments (the enzyme donor and acceptor) of the enzyme β-galactosidase are prepared by manipulation of the Z gene of the *lac* operon of the bacterium *Escherichia coli.* These two fragments spontaneously reassemble to form active enzyme, even if the enzyme donor is attached to an antigen, such as S 100A9 polypeptide. However, binding of antibody to the enzyme donor inhibits reassembly, thus blocking the formation of active enzyme and preventing enzyme activity. Thus, competition between the antigen (S1 00A9 polypeptide) and the enzyme donor antigen conjugate for a fixed quantity of antibody in the presence of the enzyme acceptor modulates the measured enzyme activity so that high concentrations of antigen produce the least inhibition of enzyme activity and the greatest measured enzyme activity, while low concentrations of antigen produce the most inhibition of enzyme activity and the least measured enzyme activity.

[01 19] Typically, however, the assay is a ELISA sandwich immunoassay as described above.

[0120] Preferably, when ELISA sandwich immunoassays are performed, 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 is at a concentration of from 75 mM to about 125 mM. More preferably, the citrate buffer is at a concentration of about 100 mM. Typically, the pH of the citrate buffer is from about pH 5 to about pH 7. Preferably, the pH of the citrate buffer is from about 5.5 to about 6.5. More preferably, the pH of the citrate buffer is about 6.0. A particularly preferred buffer is 0.1 M sodium citrate, pH 6.0.

[0121] Preferably, when ELISA sandwich immunoassays are performed, the enzyme label of the polyclonal anti-calprotectin antibody used for detection is alkaline phosphatase. However, as described above, other enzyme labels can alternatively be used.

[0122] Suitable solid supports for the performance of an ELISA sandwich immunoassay are well known in the art. However, one particularly suitable support is MaxiSorp™, produced by Nunc A/S, and described in United States Patent No. 4,980,299 to Batz et al., employing synthetic resins such as polystyrene, Luran, polypropylene, or polyvinyl chloride. Various formats are known in the art for such solid supports; one particularly suitable format is the use of a microwell plate with 96 wells. Additional solid supports are described, for example, in D. Wild & W. Kusnezow, "Separation Systems" in The Immunoassay Handbook (D. Wild, ed., 3rd ed., Elsevier, Amsterdam, 2005), ch. 10, pp. 179-1 85, incorporated herein by this reference.

[0123] Coating of the solid support with the unlabeled monoclonal anti-S1 00A9 antibody is typically performed by methods known in the art. As disclosed above, a particularly preferred buffer for coating the solid support with the unlabeled monoclonal anti-S100A9 antibody is 0.1 M sodium citrate, pH 6.0. Typically, for coating a microwell plate, the monoclonal antibody is used at a concentration of from about 1 to 4 pg/mL, preferably about 2 pg/mL, in the buffer, such as the citrate buffer described above. Preferably, the wells are covered with vapor tight adhesive plastic and stored at a

temperature from about 0° C to about 8° C, preferably about 4° C, for an incubation period of from about 6 hours to several weeks. Preferably, the incubation period is about 2 to 3 days. Alternatively, the solid support can be stored at a temperature of from about 9° C to about 37° C.

[0124] Before the performance of the ELISA sandwich immunoassay, the plates are washed to remove excess unbound antibodies. Typically, the plates are washed with a conventional washing buffer such as phosphate buffered saline. Typically, the plates are washed three to four times before the performance of the assay.

[0125] In the performance of the ELISA sandwich immunoassay, the size of the sample is typically from about 50 µl to about 150 µl. Preferably, the size of the sample is about 100 µl. The size of the sample will vary with the source of the sample and with the expected concentration of calprotectin in the sample. It is possible, and, in some cases preferable, to run the assay with multiple sample sizes, especially if there is no certain indication from the clinical condition of the patient from which was the sample was obtained about the existence of inflammatory bowel disease. As detailed below, typically, a standard curve is also constructed and the concentration of calprotectin is determined by comparison with the standard curve. In one alternative, the standard curve is constructed using a plurality of concentrations of purified calprotectin; the purified calprotectin is typically purified isolated naturally occurring calprotectin, but can also be recombinant calprotectin produced from the association of recombinantly produced S100A8 polypeptide and recombinantly produced S100A9 polypeptide. In another alternative, as detailed further below, the standard curve is constructed using a plurality of concentrations of purified S100A9 polypeptide such as the cloned recombinant S100A9 polypeptide described above.

[0126] After the samples or standards are added to the wells, the wells are covered with a suitable covering, such as plastic sheeting or film, tape or a lid and incubated at a suitable temperature, such as room temperature (20-25° C) for about 10 minutes to about 60 minutes, preferably for about 40 minutes. Preferably, incubation is performed with horizontal shaking at about 500-700 rpm. The wells are washed again as above, such as with phosphate buffered saline. To each well is then added about 50

µl to about 150 µl, preferably about 50 µl, of a labeled highly immunoaffinity purified polyclonal anti-S100A9 antibody, such as an enzyme-conjugated highly immunoaffinity purified polyclonal anti-S100A9 antibody, in a suitable buffer at a suitable dilution, and the plate is then incubated again as above. As disclosed above, the enzyme can be any type that is suitable for immunoassays. Typically, the enzyme is an enzyme that produces an optically detectable signal. A suitable enzyme is alkaline phosphatase, as discussed above. After the plates are washed again, as above, about 50 µl to about 200 µl, preferably about 100 µl, of a suitable substrate solution (e.g., p-nitrophenylphosphate if the enzyme label is alkaline phosphatase). The plate is left at room temperature for about 10 minutes to about 60 minutes, preferably about 30 minutes, at which point the color intensity (optical density) in each well is read by conventional means, such as by an ELISA reader. The concentration of calprotectin in the samples is determined by comparison of the color intensities in the respective wells with those of the standards, taking the sample dilution factor into account.

[0127] Typically, the sample is a fecal sample or a gastrointestinal (GI) tract sample, although ELISA sandwich immunoassays according to the present invention can be performed on other samples, such as whole blood, serum, plasma, urine or crevicular fluid. Testing on crevicular fluid can be used to detect or monitor the course of periodontitis (T. Nakamura et al., "The Association of Calprotectin Level in Gingival Crevicular Fluid With Gingival Index and the Activities of Collagenase and Aspartate Aminotransferase in Adult Periodontitis Patients," J. Periodontol. 71: 361-367 (2000). If 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 by Holtund et al., incorporated herein in its entirety by this reference. Briefly, this extraction procedure comprises: (1) mixing a small amount of sample (preferably 10 to 500 mg and more preferably 20-150 mg, optionally preweighed) 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; and (3) recovering the liquid extract resulting from the separation, which

contains calprotectin as well as other proteins. A suitable buffer is a Tris buffer with a pH of about 8 with added citrate. In addition to or in place of citrate, other chelators could be used. The dissociating agent can be an agent such as sodium dodecyl sulfate (SDS) 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.

[0128] In general, in immunoassays according to the present invention, either purified calprotectin or purified S100A9 protein (e.g., recombinant S100A9 protein) can be employed as the standard. In many cases, however, it is preferred to use purified S100A9 protein (e.g., recombinant S100A9 protein) as the standard.

[0129] Because calprotectin has proven to be an efficient marker for the detection or diagnosis of inflammatory bowel disease, another aspect of the invention is a method of detecting or diagnosing the presence of active inflammatory bowel disease using such immunoassays. The suggested reference limit for calprotectin for distinguishing the presence of inflammatory bowel disease from its absence is 50 mg/kg in stool samples. A value for calprotectin concentration above this limit indicates the presence of active inflammatory bowel disease, while a value for calprotectin concentration below this limit indicates the absence of active inflammatory bowel disease. Accordingly, in general, a method for diagnosing or detecting the presence of active inflammatory bowel disease comprises the steps of:

(1) providing a monoclonal anti-S100A9 antibody;

(2) coating the monoclonal anti-S100A9 antibody onto a solid support;

(3) reacting the solid support with coated monoclonal anti-S100A9 antibody with a stool sample from a patient suspected of having inflammatory bowel disease to bind any calprotectin in the sample to the solid support;

(4) washing the solid support;

(5) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support;

(6) incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody to produce a detectable signal;

(7) measuring the detectable signal to determine the concentration of calprotectin in the sample; and

(8) determining the presence or absence of active inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case active inflammatory bowel disease is present in the patient, or is less than 50 mg/kg, in which case active inflammatory bowel disease is absent in the patient.

[0130] Inflammatory bowel disease is typically a disease of relapses and remissions. Because the concentration of calprotectin in stool samples rises in relapses before the onset of clinically detectable symptoms such as pain and diarrhea, the assay of calprotectin in stool samples can be used to determine whether a patient with inflammatory bowel disease who has been in remission is at risk of suffering a relapse. In general, this method comprises the steps of:

(1) providing a monoclonal anti-S100A9 antibody;

(2) coating the monoclonal anti-S100A9 antibody onto a solid support;

(3) reacting the solid support with coated monoclonal anti-S1 00A9 antibody with a stool sample from a patient with inflammatory bowel disease that is in remission to bind any calprotectin in the sample to the solid support;

(4) washing the solid support;

(5) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support;

(6) incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody to produce a detectable signal;

(7) measuring the detectable signal to determine the concentration of calprotectin in the sample; and

(8) determining the risk of relapse of inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease exists, or is less

than 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease does not exist.

[0131] Another aspect of the present invention is a method for selection of appropriate polyclonal or monoclonal antibodies for immunoassays by testing against recombinant proteins.

[0132] In one alternative, polyclonal or monoclonal antibodies are selected by testing against recombinant S100A9 protein to select antibodies with high binding affinity to S100A9 polypeptide. In general, such a selection procedure comprises the steps of:

(1) providing a plurality of polyclonal or monoclonal antibodies binding S100A9 protein;

(2) separately reacting each of the polyclonal or monoclonal antibodies with recombinant S100A9 protein under conditions where equilibrium is reached;

(3) determining the affinity of each of the polyclonal or monoclonal antibodies for S100A9 polypeptide; and

(4) selecting the polyclonal or monoclonal antibody with the highest affinity for S100A9 polypeptide.

[0133] The antibodies to be selected for high affinity can be polyclonal antibodies or monoclonal antibodies. However, in a single selection procedure, either polyclonal antibodies or monoclonal antibodies should be selected; in other words, polyclonal antibodies and monoclonal antibodies should be selected in different selection procedures.

[0134] Methods for determining the affinity of a homogeneous or virtually homogeneous antibody for a homogeneous antigen that possesses one epitope bound by the antibody are well known in the art. A particularly useful method is to use radiolabeled antigen in equilibrium with antibody and then separate the bound from the free antigen, quantitating the bound and the free antigen separately. This can be done, for example, by precipitating the antibody. Methods of precipitating the antibody are well known in the art and include, for example, the use of ammonium sulfate or polyethylene glycol, precipitation with Protein A, or precipitation with a second antibody

specific for a determinant on the antibody. The affinity is then determined in units of liters per mole, or M^{-1}, such as by the use of a Scatchard plot.

[0135] The affinity of the antibody to be measured can then be compared with the affinity of a standard antibody for S100A9 polypeptide, such as the monoclonal antibody designated as Calpro Mab CAL1-4H1/2/2. Typically, an antibody suitable for use in immunoassays for calprotectin has an affinity of at least 80% of the affinity of Calpro Mab CAL1-4H1/2/2. Preferably, an antibody suitable for use in immunoassays for calprotectin has an affinity of at least 90% of the affinity of Calpro Mab CAL1-4H1/2/2. More preferably, an antibody suitable for use in immunoassays for calprotectin has an affinity of at least 95% of the affinity of Calpro Mab CAL1-4H1/2/2. Most preferably, an antibody suitable for use in immunoassays for calprotectin has an affinity of at least 97.5% of the affinity of Calpro Mab CAL1-4H1/2/2.

[0136] Similar affinity measurements for selection of suitable antibodies can be performed for antibodies specifically binding S100A8 and S100A12 polypeptides. The amino acid sequence of human S100A8 is known and is MLTELEKALNSIIDVYHKYSLIKGNFHAVYRDDLKKLLETECPQYIRKKGADVWFKELDI NTDGAVNFQEFLILVIKMGVAAHKKSHEESHKE (SEQ ID NO: 4). The sequence of human DNA encoding S100A8 is ATGTCTCTTGTCAGCTGTCTTTCAGAAGACCTGGTGGGGCAAGTCCGTGGGCATC ATGTTGACCGAGCTGGAGAAAGCCTTGAACTCTATCATCGACGTCTACCACAAGTA CTCCCTGATAAAGGGGAATTTCCATGCCGTCTACAGGGATGACCTGAAGAAATTGC TAGAGACCGAGTGTCCTCAGTATATCAGGAAAAAGGGTGCAGACGTCTGGTTCAA AGAGTTG GATATCAACACTG ATG GTGCAGTTAACTTCCAG GAGTTCCTCATTCTG G TGATAAAGATGGGCGTGGCAGCCCACAAAAAAAGCCATGAAGAAAGCCACAAAGA GTAGCTGAGTTACTGGGCCCAGAGGCTGGGCCCCTGGACATGTACCTGCAGAATA ATAAAGTCATCAATACCTCAAAAAAAAAAAAAAAAAAAA (SEQ ID NO: 5). The sequence of SEQ ID NO: 5 is derived from the mRNA and therefore excludes introns possibly present in the genomic sequence. A suitable DNA sequence for expression of S100A8 in *Escherichia coli* is GGTACCATATGCTGACCGAACTGGAAAAAGCGCTGAACAGCATCATTGATGTGTAC

CACAAATATAGCCTGATCAAAGGTAACTTTCATGCGGTGTATCGTGATGATCTGAA
AAAACTGCTGGAAACCGAATGCCCGCAGTATATTCGTAAAAAAGGCGCGGATGTG
TGGTTTAAAGAACTGGATATCAACACCGATGGCGCGGTGAACTTTCAGGAATTTCT
GATCCTGGTTATCAAAATGGGCGTGGCGGCGCATAAAAAAAGCCATGAAGAAAGC
CACAAAGAATGATAACTCGAGCTC (SEQ ID NO: 6). This sequence has been optimized for expression in *E. coli* and has a codon adaptation index of 0.97.

[0137] SEQ ID NO: 6 is only one example of a DNA sequence that can be used to express the amino acid sequence of SEQ ID NO: 4. Other DNA sequences can readily be constructed by one of ordinary skill in the art employing the known degeneracy of DNA codons in the genetic code. In general, any DNA sequence encoding the amino acid sequence of SEQ ID NO: 4 can be used, although, in some cases, particular DNA sequences are preferred because of codon utilization biases in some organisms used for expression, such as *Escherichia coli* or because of the presence of negative c/s-acting sites such as internal RBS sites, TATA boxes, or Chi recombination sites, which may negatively influence expression. The DNA sequence of SEQ ID NO: 6 is optimized for expression in *E. coli,* with a CAI (codon adaptation index) of 0.97, which indicates that the sequence is extremely well adapted for expression in E. *coli.* Other sequences can be used to express S100A8 in E. *coli* or in other organisms, including eukaryotic cells such as yeast cells or mammalian cells.

[0138] The amino acid sequence of human S100A12 is known and is MTKLEEHLEGIVNIFHQYSVRKGHFDTLSKGELKQLLTKELANTIKNIKDKAVIDEIFQGL DANQDEQVDFQEFISLVAIALKAAHYHTHKE (SEQ ID NO: 7). The genomic DNA sequence of human S100A12 is
ACCACTGCTGGCTTTTTGCTGTAGCTCCACATTCCTGTGCATTGAGGGGTTAACAT
TAGGCTGGGAAGATGACAAAACTTGAAG AGCATCTG GAGGGAATTGTCAATATCTT
CCACCAATACTCAGTTCG GAAGGGGCATTTTG ACACCCTCTCTAAG GGTGAGCTG
AAGCAGCTGCTTACAAAGGAGCTTGCAAACACCATCAAGAATATCAAAGATAAAGC
TGTCATTGATGAAATATTCCAAGGCCTGGATGCTAATCAAGATGAACAGGTCGACT
TTCAAGAATTCATATCCCTGGTAGCCATTGCGCTGAAGGCTGCCCATTACCACACC
CACAAAGAGTAGGTAGCTCTCTGAAGGCTTTTTACCCAGCAATGTCCTCAATGAGG

GTCTTTTCTTTCCCTCACCAAAACCCAGCCTTGCCCGTGGGGAGTAAGAGTTAATA AACACACTCACGAAAAGTT (SEQ ID NO: 8). The sequence of SEQ ID NO: 8 is derived from mRNA and therefore excludes introns possibly present in the genomic sequence. Suitable sequences for the expression of S100A12 can be constructed for expression in *E. coli* or in eukaryotic cells.

[0139] Another aspect of the invention is the selection of polyclonal or monoclonal antibodies against S100A9 polypeptide in order to select antibodies that have the lowest possible cross-reactivity against either S100A8 polypeptide, S100A12 polypeptide, or both. In general, such a method comprises:

(1) providing a polyclonal or monoclonal antibody binding S100A9 polypeptide;

(2) reacting the polyclonal or monoclonal antibody with recombinant S100A9 polypeptide under conditions where equilibrium is reached; and

(3) determining the affinity of the polyclonal or monoclonal antibody for S100A9 polypeptide;

(4) reacting the polyclonal or monoclonal antibody with a recombinant polypeptide selected from the group consisting of recombinant S100A8 polypeptide and recombinant S100A12 polypeptide under conditions where equilibrium is reached;

(5) determining the affinity of the polyclonal or monoclonal antibody for the recombinant polypeptide selected from the group consisting of recombinant S100A8 polypeptide and recombinant S100A12 protein;

(6) comparing the affinity of the polyclonal or monoclonal antibody for recombinant S100A9 polypeptide and for the recombinant polypeptide selected from the group consisting of recombinant S100A8 polypeptide and recombinant S100A12 polypeptide to determine the cross-reactivity of the polyclonal or monoclonal antibody with the recombinant polypeptide selected from the group consisting of recombinant S100A8 polypeptide and recombinant S100A12 polypeptide; and

(7) selecting the polyclonal or monoclonal antibody with the lowest cross-reactivity with the recombinant polypeptide selected from the group consisting of recombinant S100A8 polypeptide and recombinant S100A12 polypeptide.

[0140] Typically, an antibody suitable for use in immunoassays for calprotectin has a cross-reactivity, defined as the ratio of the affinity of the antibody for the recombinant polypeptide selected from the group consisting of recombinant S100A8 polypeptide and recombinant S100A12 polypeptide to the affinity of the antibody for recombinant S100A9 polypeptide, of no greater than about 0.10. Preferably, the antibody has a cross-reactivity no greater than about 0.05. More preferably, the antibody has a cross-reactivity no greater than about 0.02.

[0141] Another aspect of the present invention is a method of immunoaffinity purification of polyclonal anti-calprotectin antibodies to remove antibodies against epitopes present on standards but not on calprotectin in actual stool samples. In general, such a method comprises the steps of:

(1) providing a polyclonal anti-S100A9 antibody;

(2) performing immunoaffinity purification with an affinity ligand selected from the group consisting of a recombinant calprotectin subunit and a related S100 protein so that antibody molecules binding the affinity ligand are retained by the affinity ligand; and

(3) isolating a fraction of polyclonal antibody not retained by the affinity ligand in order to remove antibodies against epitopes present on standards but not on calprotectin in actual stool samples.

[0142] The affinity ligand selected from the group consisting of a recombinant calprotectin subunit and a related S100 protein can be a recombinant calprotectin subunit, such as the S100A8 subunit. Alternatively, the affinity ligand can be a related S100 protein, such as the S100A12 protein. Other S100 proteins, such as the S100A1 protein, the S100A2 protein, the S100A3 protein, the S100A4 protein, the S100A5 protein, the S100A6 protein, the S100A7 protein, the S100A10 protein, the S100A11 protein, the S100A12 protein, the S100A13 protein, the S100A14 protein, the S100A15 protein, and the S100A16 protein, can alternatively be used as the affinity ligand.

[0143] Similarly, another aspect of the present invention is a method for the selection and removal of anti-calprotectin antibodies cross-reacting with a S100 protein other than S100A8 or S100A9. In general, this method comprises:

(1) providing a polyclonal anti-S100A9 antibody;

(2) performing immunoaffinity purification with at least one affinity ligand that is a S100 protein other than S100A8 or S100A9; and

(3) isolating a fraction of polyclonal antibody not retained by the affinity ligand in order to remove anti-calprotectin antibodies that cross-react with at least one S100 protein other than S100A8 or S100A9.

[0144] In this procedure, immunoaffinity purification can be performed using multiple ligands in the same immunoaffinity column. For example, immunoaffinity purification can be performed employing 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14 of the S100 proteins other than S100A8 or S100A9 in a single immunoaffinity column. Alternatively, immunoaffinity purification can be performed using multiple immunoaffinity columns in succession, each immunoaffinity column having one or more than one ligand. When multiple affinity columns are employed, the output from the first column is then applied to the next column, and this is repeated as many times as desired.

[0145] Methods for immunoaffinity chromatography are well known in the art and are described, for example, in G.T. Hermanson, "Bioconjugate Techniques" (Academic Press, San Diego, 1996), pp. 484-485, incorporated herein by this reference.

[0146] Yet another aspect of the invention is a method for the selection of anti-S100A9 antibody preparations that give correct readings and provide a wide assay range in the ELISA. This method is based on the findings described above that many antibody preparations provide falsely low readings for calprotectin concentrations in actual stool samples. Therefore, in general, such a method for the selection of antibody preparations comprises:

(1) providing an unlabeled monoclonal anti-S100A9 antibody and an enzyme-labeled polyclonal anti-S100A9 antibody as described above that have been shown to give accurate results in an ELISA assay with stool samples;

(2) providing a candidate preparation of unlabeled monoclonal anti-S100A9 antibody;

(3) providing a candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody;

(4) performing an ELISA sandwich immunoassay using the unlabeled monoclonal anti-S100A9 antibody and the enzyme-labeled polyclonal anti-S100A9 antibody of (1) using a plurality of stool samples;

(5) performing an ELISA sandwich immunoassay using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody;

(6) comparing the results from the ELISA sandwich immunoassays of (4) and (5) to determine whether the results are substantially equivalent; and

(7) selecting candidate preparations of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that produce substantially equivalent results on the plurality of stool samples.

[0147] As used herein, the term "substantially equivalent," as used with respect to the results obtained from performing ELISA assays with the pairs of antibodies (the unlabeled monoclonal anti-S100A9 antibody and the labeled polyclonal anti-S100A9 antibody), means that the results obtained using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody differ from the results obtained using a preparation of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that have been shown to give accurate results on stool samples vary by no more than about 10% for any stool sample tested. Preferably, the results vary by no more than about 5% for any stool sample tested.

[0148] Yet another aspect of the present invention is a method of performing an immunoassay for multiple antigens to achieve a more sensitive result. Typically, the antigens are S100A9 and the related protein S100A12, and, in such assays, a positive result is obtained if an elevated concentration of at least one antigen exists in the sample.

[0149] In general, such a method comprises the steps of:

(1) providing a stool sample from a subject;

(2) performing an ELISA sandwich immunoassay for S100A9 using an unlabeled monoclonal anti-S100A9 antibody and an enzyme-labeled polyclonal anti-calprotectin antibody;

(3) performing an immunoassay for a related antigen;

(4) determining the concentration of calprotectin in the stool sample from the results of the ELISA sandwich immunoassay for S100A9;

(5) determining the concentration of the related antigen in the stool sample from the results of the immunoassay for the related antigen; and

(6) comparing the concentration of S100A9 polypeptide and the concentration of the related antigen in the stool sample with the cutoff values for S100A9 polypeptide and the related antigen to determine whether or not inflammatory bowel disease exists in the subject.

[0150] As described above, the cutoff value for calprotectin is 50 mg/kg.

[0151] As used herein, the term "related antigen" means that the antigen in question is one that is associated with the occurrence of inflammatory bowel disease and can be used to discriminate between patients who have inflammatory bowel disease and patients who do not have inflammatory bowel disease. The term "related antigen" does not necessarily imply that the antigen in question necessarily has any defined degree of sequence identity or sequence similarity with S100A9, although a degree of sequence identity or sequence similarity may in fact exist.

[0152] In one alternative, the related antigen is S100A12. A suitable cutoff value for S100A12 is 66 pg/kg (T. Kaiser et al,. "Faecal S100A12 as a Non-invasive Marker Distinguishing Inflammatory Bowel Disease from Irritable Bowel Syndrome," Gut 56:1706-13 (2007)). Typically, if the related antigen is S100A12, the immunoassay is an ELISA sandwich immunoassay; the ELISA sandwich immunoassay for S100A12 can employ an unlabeled monoclonal anti-A12 antibody and an enzyme-labeled polyclonal anti-S100A12 antibody. However, other alternatives are possible for the unlabeled capture antibody and the labeled antibody in an ELISA immunoassay. Additionally, the immunoassay for the related antigen, such as S100A12, can be an immunoassay other than an ELISA immunoassay.

[0153] In another alternative, the related antigen is lactoferrin. Immunoassays for lactoferrin are well known in the art and are described, for example, in U.S. Patent No. 7,560,240 to Boone et al, U.S. Patent No. 7,192,724 to Boone et al., U.S. Patent 6,727,073 to Moore et al., U.S. Patent No. 6,727,073 to Moore et al., U.S. Patent No. 6,1 74,664 to Heine, U.S. Patent No. 5,552,292 to Uchida et al., U.S. Published Patent Application No. 2009/02531 55 by Boone et al., U.S. Published Patent Application No. 2009/0053736 by Mattingly et al., U.S. Published Patent Application No. 2008/01 6671 9 by Lois, U.S. Published Patent Application No. 2008/0085524 by Lois, U.S. Published Patent Application No. 2006/0003392 by Oh et al., and U.S. Published Patent Application No. 2004/01 37536 by Boone et al. Methods for assay of lactoferrin as known in the art can include sandwich immunoassays such as ELISA immunoassays and lateral flow immunoassays, as well as competitive immunoassays. A suitable cutoff value for lactoferrin is 2.2 ng/mL.

[0154] The immunoassay for the related antigen can be performed by a competitive immunoassay method such as a competitive immunoassay on a solid phase platform. The immunoassay for the related antigen can also be another conventional competitive immunoassay, such as a homogeneous or heterogeneous competitive immunoassay, or a sandwich immunoassay such as a conventional ELISA or a conventional lateral flow immunoassay. Immunoassays are described in D. Wild, ed., "The Immunoassay Handbook" (3rd ed., Elsevier, Amsterdam, 2005). General considerations are described in C. Davies, "Introduction to Immunoassay Principles" in "The Immunoassay Handbook,". (D. Wild, ed., 3rd Ed., Elsevier, Amsterdam, 2005), ch. 1, pp. 3-40, incorporated herein by this reference.

[0155] Another aspect of the present invention is a kit for immunoassay of S100A9 and diagnosis, screening, or monitoring of inflammatory bowel disease. The kit can comprise, separately packaged:

(1) a quantity of an unlabeled monoclonal anti-S1 00A9 antibody suitable for performance of an ELISA sandwich immunoassay for the detection of S100A9 polypeptide; and

(2) a quantity of an enzyme-labeled polyclonal anti-calprotectin antibody suitable for performance of an ELISA sandwich immunoassay for the detection of S100A9 polypeptide.

[0156] Typically, the unlabeled monoclonal anti-S100A9 antibody is in citrate buffer, as described above.

[0157] The quantities of the unlabeled monoclonal anti-S100A9 antibody and the enzyme-labeled polyclonal anti-calprotectin antibody can be such that they are intended to be used in a single assay. Alternatively, the quantities can be such that they are intended to be used in a plurality of individual assays, such as 2, 3, 5, 10, 15, 20, 25, 30, 40, 50, or 100 individual assays, and the appropriate quantity for a single assay measured out by the individual performing the assay.

[0158] In addition, a kit according to the present invention can further include one or more of the following items, all separately packaged:

(1) a solid support suitable for performance of the ELISA immunoassay;

(2) wash buffer for washing the solid support after coating the solid support with immobilized antibody and after applying the sample to the solid support;

(3) substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody; and

(4) a set of S100A9 standards of differing concentrations so that a standard curve for S100A9 can be established.

[0159] As indicated above, in another alternative of an immunoassay method according to the present invention, a lateral flow platform is used.

[0160] In an immunoassay employing a lateral flow platform as described above, the assay platform is typically constructed of a solid support that provides lateral flow of a sample through the assay platform when a sample is applied to a sampling platform that is in operable contact with the assay platform. The sampling platform and the assay platform are typically constructed of a material such as nitrocellulose, glass fiber, paper, nylon, polyvinylidene fluoride, or a synthetic nanoporous polymer. Suitable materials are well known in the art and are described, for example, in U.S. Patent No. 7,256,053 to Hu, 7,214,417 to Lee et al., 7,238,538 to Freitag et al., 7,238,322 to Wang

et al., 7,229,839 to Thayer et al., 7,226,793 to Jerome et al., RE39,664 to Gordon et al., 7,205,1 59 to Cole et al., 7,1 89,522 to Esfandiari, 7,186,566 to Qian, 7,1 66,208 to Zweig, 7,144,742 to Boehringer et al., 7,132,078 to Rawson et al., 7,097,983 to Markovsky et al., 7,090,803 to Gould et al., 7,045,342 to Nazareth et al., 7,030,21 0 to Cleaver et al., 6,981 ,522 to O'Connor et al., 6,924,1 53 to Boehringer et al., 6,849,414 to Guan et al., 6,844,200 to Brock, 6,841 ,1 59 to Simonson, 6,767,714 to Nazareth et al., 6,699,722 to Bauer et al., 6,656,744 to Pronovost et al., 6,528,323 to Thayer et al., 6,297,020 to Brock, 6,140,1 34 to Rittenburg, 6,1 36,61 0 to Polito et al., 5,965,458 to Kouvonen et al., 5,71 2,1 70 to Kouvanen et al., 4,956,302 to Gordon et al., and 4,943,522 to Eisinger et al., all of which are incorporated herein by this reference.

[0161] Various alternatives for the construction of the lateral flow platform and device and the performance of the sandwich lateral flow assay for S 100A9 polypeptide are known in the art and are described below. Common to all of these platforms, devices, and assay methods is the immobilization of an unlabeled anti-calprotectin antibody on the assay platform. Because this is a sandwich immunoassay, both a mobile labeled anti-calprotectin antibody and any calprotectin in the sample need to migrate to the immobilized unlabeled anti-S1 00A9 antibody. Any calprotectin in the sample will then react with both the immobilized unlabeled anti-S1 00A9 antibody and the mobile labeled anti-calprotectin antibody forming an antibody-antigen-antibody "sandwich." The quantity of labeled anti-calprotectin antibody bound to the S 100A9 polypeptide in the sample and then to the immobilized unlabeled anti-S1 00A9 antibody accurately reflects the concentration of S100A9 polypeptide, and thus calprotectin, in the sample. Therefore, these platforms, devices, and assay methods employ a solid support that provides lateral flow. The solid support that provides lateral flow has a first end and a second end. The immobilization occurs at a defined region of the solid support that provides lateral flow, the defined region being referred to herein as the "detection zone." Typically, the detection zone is located between the first end and the second end of the solid support that provides lateral flow, closer to the second end. As detailed below, the sample and the labeled anti-calprotectin antibody are typically applied at or near the first end of the solid support that provides lateral flow so that the

sample and the labeled anti-calprotectin antibody migrate through the support to the detection zone. Various methods can be used to do this, again, as detailed below. This sandwich lateral flow assay is an assay in which the quantity of label bound at the detection zone is directly proportional to the quantity of S100A9 polypeptide, and thus calprotectin, in the sample. Lateral flow immunoassay devices are further described in R. Wong & H. Tse, eds., "Lateral Flow Immunoassay" (Humana Press, New York, NY, 2009).

[0162] In Alternative I for the lateral flow platform and device, the labeled anti-calprotectin antibody is located in the device before the sample is applied. The labeled anti-calprotectin antibody is dried down in the device in a position where it is contacted by sample applied to the device, such that the labeled anti-calprotectin antibody is then solubilized and mobilized by the sample contacting the dried labeled anti-calprotectin antibody. The dried labeled anti-calprotectin antibody can be located, for example, in: (a) a conjugate zone located on the solid support that provides lateral flow at a position where the sample first contacts the dried labeled anti-calprotectin antibody; (b) a conjugate pad in direct or indirect operable contact with one end of the solid support that provides lateral flow such that the end is distal to the portion of the solid support that provides lateral flow in which the detection zone is located; or (c) a conjugate pad located in another position that is not initially in direct or indirect operable contact with the solid support that provides lateral flow but is moved into direct or indirect operable contact with the solid support that provides lateral flow after the commencement of the assay.

[0163] In Alternative I, the sample can be applied in a number of locations; in each case, the sample must flow in a path that results in solubilization and mobilization of the dried labeled anti-calprotectin antibody. For example, the sample can be applied to the conjugate pad, if a conjugate pad is used. This will result in solubilization and mobilization of the dried labeled anti-calprotectin antibody. Alternatively, the sample can be applied to a sample pad that is either located so that it is in either direct or indirect operable contact with either the solid support that provides lateral flow (in alternatives in which the dried labeled anti-calprotectin antibody is located in a

conjugate zone directly on the solid support) or the conjugate pad, or located so that it is moved into either direct or indirect operable contact with either the solid support that provides lateral flow (in alternatives in which the dried labeled anti-calprotectin antibody is located in a conjugate zone directly on the solid support) or the conjugate pad.

[0164] In Alternative II, the labeled anti-calprotectin antibody is not present in the device prior to sample application. In Alternative II, labeled anti-calprotectin antibody, typically already mobile and in solution, is added to the sample just before application to the device. In Alternative II, the sample and the mobile labeled anti-calprotectin antibody can be applied to: (a) a portion of the solid support that provides lateral flow that is separated from the detection zone; or (b) a sample/conjugate pad that is in either direct or indirect operable contact with the end of the solid support that provides lateral flow separated from the detection zone when the sample and labeled anti-calprotectin antibody is applied thereto, or is moved into direct or indirect operable contact with the end of the solid support that provides lateral flow separated from the detection zone after the application of the sample and the labeled anti-calprotectin antibody.

[0165] Typically, the solid support that provides lateral flow is backed with a nonporous backing on one side, such as a plastic strip. The solid support that provides lateral flow can also be enclosed in a casing that provides openings as required for the performance of the assay. Depending upon the exact configuration of the assay device, the openings can include an opening for application of the sample and/or the labeled anti-calprotectin antibody, an opening positioned for detection at the detection zone, and an opening positioned for detection at a control zone, as described below.

[0166] The device can include a control zone to verify that the assay has been performed correctly. In one alternative, the control zone binds the labeled anti-calprotectin antibody. The control zone is typically located close to the detection zone but separated from it. In one alternative, the control zone includes an immobilized antibody that binds the labeled anti-calprotectin antibody by binding to a portion of the antibody distinct from the paratope of the labeled anti-calprotectin antibody, such as, but not limited to, an epitope located in the constant region of the labeled anti-calprotectin antibody. The binding of the labeled anti-calprotectin antibody to the immobilized

S100A9 polypeptide in the detection zone should not interfere with the binding of the labeled anti-calprotectin antibody to the control zone, and the binding of the labeled anti-calprotectin antibody to the control zone should not interfere with the binding of the labeled anti-calprotectin antibody to the immobilized S100A9 polypeptide in the detection zone. If this version of a control zone is used, additional labeled anti-calprotectin antibody should be included to account for the quantity of labeled anti-calprotectin antibody expected to be bound to the control zone.

[0167] In yet another alternative, the control zone can include immobilized S100A9 polypeptide or other appropriate polypeptide, which directly binds the labeled anti-calprotectin antibody. If this version of a control zone is used, additional labeled anti-calprotectin should again be included to account for the quantity of anti-calprotectin antibody expected to be bound to the control zone.

[0168] In another alternative, the control zone includes an unrelated antigen and the device includes a second labeled antibody that specifically binds the unrelated antigen. There should be substantially no cross-reactivity between the labeled anti-calprotectin antibody and the second labeled antibody that specifically binds the unrelated antigen. The labeled antibody that specifically binds the unrelated antigen is typically present in, or added to, the device along with the labeled anti-calprotectin antibody. The label of the labeled antibody that specifically binds the unrelated antigen can be the same as the label of the anti-calprotectin antibody, or can be a different label; typically, however, for convenience in reading, the label of the labeled antibody that specifically binds the unrelated antigen is typically the same as the label of the anti-calprotectin antibody.

[0169] A number of alternatives for an assay device that can perform such sandwich immunoassays are depicted in Figures 1A, 1B, 2A, 2B, 3A, 3B, 4, 5, 6A, 6B, 7A, 7B, and 8.

[0170] Figure 1A depicts a top view of a first alternative for an assay device that can perform a sandwich immunoassay according to Alternative I as described above. In Figure 1A, the device 10 includes a solid support that provides lateral flow 12 having a first end 14 and a second end 16. The solid support that provides lateral flow 12 has a

detection zone 18 and an optional control zone 20. The detection zone 18 and, if present, the optional control zone 20 are located closer to the second end 16 of the solid support that provides lateral flow 12 than to the first end 14 of the solid support that provides lateral flow 12; if present, the optional control zone 20 can be either located closer to the second end 16 of the solid support that provides lateral flow 12 than the detection zone 18, or the detection zone 18 can be located closer to the second end 16 of the solid support that provides lateral flow 12 than the control zone 20. The solid support that provides lateral flow 12 also has a conjugate zone 22; the conjugate zone is located closer to the first end 14 of the solid support that provides lateral flow than to the second end 16. The device 10 includes a sample pad 24 for application of the sample that is in operable contact with the solid support that provides lateral flow 12 so that the sample flows from the sample pad 24 into the solid support that provides lateral flow 12 so that the labeled antibody at the conjugate zone 22 is solubilized and mobilized. As shown in Figure 1B, the device 10 can optionally include a backing 26 for the solid support that provides lateral flow 12 and a casing 28. The casing 28 has apertures, including a first aperture 30 for application of the sample to the sample pad 20, a second aperture 32 allowing viewing and/or measurement of labeled anti-calprotectin antibody bound to the detection zone 18, and a third aperture 34 allowing viewing and/or measurement of labeled anti-S1 00A9 antibody or other antibody used as a control bound to the control zone 20.

[0171] Figure 2A depicts a top view of a second alternative for an assay device that can perform a sandwich immunoassay according to Alternative I as described above. In Figure 2A, the device 40 includes a solid support that provides lateral flow 42 having a first end 44 and a second end 46. The solid support that provides lateral flow has a detection zone 48 and an optional control zone 50. The device 40 has a conjugate pad 52 in operable contact with the first end 44 of the solid support that provides lateral flow 42; sample is applied to the conjugate pad 52 so that the labeled antibody in the conjugate pad 52 is solubilized and mobilized. As shown in Figure 2B, the device 40 can optionally include a backing 54 for the solid support that provides lateral flow 42 and a casing 56. The casing 56 has apertures, including a first aperture

58 for application of the sample to the conjugate pad 52, a second aperture 60 allowing viewing and/or measurement of labeled anti-calprotectin antibody bound to the detection zone 48, and a third aperture 62 allowing viewing and/or measurement of labeled anti-calprotectin antibody or other antibody used as a control bound to the control zone 50.

[0172] Figure 3A depicts a top view of a third alternative for an assay device that can perform a sandwich immunoassay according to Alternative I as described above. In Figure 3A, the device 100 includes a solid support that provides lateral flow 102 having a first end 104 and a second end 106. The solid support that provides lateral flow 102 has a detection zone 108 and an optional control zone 110. The device 100 has a conjugate pad 112 in operable contact with the first end 104 of the solid support that provides lateral flow 102 and a sample pad 114 in operable contact with the conjugate pad 112; sample is applied to the sample pad 114 and flows to the conjugate pad 112 so that the labeled antibody in the conjugate pad 112 is solubilized and mobilized. As shown in Figure 3B, the device 100 can optionally include a backing 116 for the solid support that provides lateral flow 102 and a casing 118. The casing 118 has apertures, including a first aperture 120 for application of the sample to the sample pad 114, a second aperture 122 allowing viewing and/or measurement of labeled anti-calprotectin antibody bound to the detection zone 108, and a third aperture 124 allowing viewing and/or measurement of labeled anti-calprotectin antibody or other antibody used as a control bound to the control zone 110.

[0173] Figure 4 depicts a fourth alternative for an assay device that can perform a sandwich immunoassay according to Alternative I as described above. The device of Figure 4 is a folding device that is constructed of two substantially planar parts connected by a hinge. The device 140 includes a solid support that provides lateral flow 142. The solid support that provides lateral flow 142 has a first end 144 and a second end 146, with a detection zone 148 and, optionally, a control zone 150. The solid support that provides lateral flow 142 is attached to a first substantially planar part 152. The device 140 further includes an applicator part that is a second substantially planar part 154; a hinge 156 flexibly connects the applicator part that is a second substantially

planar part 154 and the first substantially planar part 152 that includes the support that provides lateral flow 142. The applicator part that is a second substantially planar part 154 includes a conjugate pad 158. In use, sample is added to the conjugate pad 158 so that the labeled antibody in the conjugate pad 158 is solubilized and mobilized. The device 140 is then closed via the hinge 156 so that the conjugate pad 158 is brought into operable contact with the first end 144 of the solid support that provides lateral flow 142 so that the sample and the labeled antibody flow through the solid support that provides lateral flow 142.

[0174] Figure 5 depicts a fifth alternative for an assay device that can perform a sandwich immunoassay according to Alternative I as described above. The device of Figure 5 is also a folding device that is constructed of two substantially planar parts connected by a hinge; it is generally similar to that of Figure 4, except that the applicator part includes a separate sample pad and a conjugate pad. The device 200 includes a solid support that provides lateral flow 202. The solid support that provides lateral flow 202 has a first end 204 and a second end 206, with a detection zone 208 and, optionally, a control zone 210. The solid support that provides lateral flow 202 is attached to a first substantially planar part 212. The device 200 further includes an applicator part that is a second substantially planar part 214; a hinge 216 flexibly connects the applicator part that is a second substantially planar part 214 and the first substantially planar part 212 that includes the support that provides lateral flow 202. The applicator part that is a second substantially planar part 214 includes a sample pad 218 and a conjugate pad 220; the sample pad 218 is in operable contact with the conjugate pad 220. In use, sample is added to the sample pad 218; the sample then flows to the conjugate pad 220 so that the labeled antibody in the conjugate pad 220 is solubilized and mobilized. The device 200 is then closed via the hinge 216 so that the conjugate pad 220 is brought into operable contact with the first end 204 of the solid support that provides lateral flow 202 so that the sample and the labeled antibody flow through the solid support that provides lateral flow 202.

[0175] Figure 6A is a top view of a first alternative for an assay device that can perform a sandwich immunoassay according to Alternative II as described above. In

Alternative II, the labeled anti-calprotectin antibody is not present on the device at the start of the assay, but is added to the device together with the sample at the commencement of the assay. In the device of Figure 6A, the device 300 includes a solid support that provides lateral flow 302 having a first end 304 and a second end 306. The solid support that provides lateral flow 302 has a detection zone 308 and an optional control zone 310. The device 300 includes a sample pad 312 for application of the sample and the labeled anti-calprotectin antibody that is in operable contact with the solid support that provides lateral flow 302 so that the sample and the labeled anti-calprotectin antibody flow from the sample pad 312 into the solid support that provides lateral flow 302. As shown in Figure 6B, the device 300 can optionally include a backing 314 for the solid support that provides lateral flow 302 and a casing 316. The casing 316 has apertures, including a first aperture 318 for application of the sample and the labeled anti-calprotectin antibody to the sample pad 312, a second aperture 320 allowing viewing and/or measurement of labeled anti-calprotectin antibody bound to the detection zone 308, and a third aperture 322 allowing viewing and/or measurement of labeled anti-calprotectin antibody or other antibody used as a control bound to the control zone 310.

[0176] Figure 7A is a top view of a second alternative for an assay device that can perform a sandwich immunoassay according to Alternative II as described above. The device of Figure 7A is generally similar to that of Figure 6A, but the sample pad is dispensed with and the sample and labeled anti-calprotectin antibody are applied directly to the first end of the solid support that provides lateral flow. In the device of Figure 7A, the device 340 includes a solid support that provides lateral flow 342 having a first end 344 and a second end 346. The solid support that provides lateral flow 342 has a detection zone 348 and an optional control zone 350. The device 340 is arranged so that the sample and the labeled anti-calprotectin antibody are applied directly to the first end 344 of the solid support that provides lateral flow 342. As shown in Figure 7B, the device 340 can optionally include a backing 352 for the solid support that provides lateral flow 342 and a casing 354. The casing 354 has apertures, including a first aperture 356 for application of the sample and the labeled anti-calprotectin antibody to

the first end 344 of the solid support that provides lateral flow 342, a second aperture 358 allowing viewing and/or measurement of labeled anti-calprotectin antibody bound to the detection zone 348, and a third aperture 360 allowing viewing and/or measurement of labeled anti-calprotectin antibody or other antibody used as a control bound to the control zone 350.

[0177] Figure 8 is a third alternative for an assay device that can perform a sandwich immunoassay according to Alternative II as described above. The device of Figure 8 is a folding device that is constructed of two substantially planar parts connected by a hinge. The device 400 includes a solid support that provides lateral flow 402. The solid support that provides lateral flow 402 has a first end 404 and a second end 406, with a detection zone 408 and, optionally, a control zone 410. The solid support that provides lateral flow 402 is attached to a first substantially planar part 412. The device 400 further includes a applicator part 414 that is a second substantially planar part; a hinge 416 flexibly connects the applicator part 414 that is a second substantially planar part and the first substantially planar part 412 that includes the solid support that provides lateral flow 402. The applicator part 414 includes a sample pad 418. In use, sample and labeled anti-calprotectin antibody are added to the sample pad 418. The device 400 is then closed via the hinge 416 so that the sample pad 418 is brought into operable contact with the first end 404 of the solid support that provides lateral flow 402 so that the sample and the labeled antibody flow through the solid support that provides lateral flow 402.

[0178] Yet another alternative for an assay device that can perform a sandwich immunoassay according to Alternative II as described above is constructed according to Figure 5 above, under Alternative I, except that the conjugate pad 220 of Figure 5 is replaced by a conjugate application pad to which labeled antibody is applied at the commencement of the assay. The assay then proceeds in such a way that the sample migrates to the conjugate application pad, picking up antibody, and then to the detection zone.

[0179] Other assay devices can be constructed on similar principles for performing sandwich immunoassays for S100A9 polypeptide. These devices can, for

example, include additional elements for controlling flow between components. It is also possible to construct multiplex devices operating on similar principles for assay of multiple samples and/or standards in a single assay.

[0180] When an immunoassay is performed on a device for performing a lateral flow sandwich immunoassay according to the present invention, reading the result can be done either by eye, for a qualitative estimate of the concentration of the analyte being assayed, such as S100A9 polypeptide, or by a reader for a lateral flow immunoassay device. Readers for lateral flow immunoassay devices are known in the art and are described, for example, in B. O'Farrell, "Evolution in Lateral Flow-Based Immunoassay Systems" in Lateral Flow Immunoassay (R. Wong & H. Tse, eds., Humana Press, New York, NY, 2009), ch.1 , pp. 23-28, incorporated herein by this reference. The choice of a reader will depend on the label of the labeled anti-calprotectin antibody and the signal produced by that label; however, readers include charge coupled device (CCD) cameras, confocal or other optical sensors for detection of fluorescent particles subsequent to LED excitation, and detection of paramagnetic monodisperse latexes using magnetic assay reader technology. Other reader systems are known in the art.

[0181] In general, therefore, another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 polypeptide comprising a solid support for lateral flow, a labeled anti-calprotectin antibody that is either present on the device before the commencement of the immunoassay or is added to the device at the commencement of the immunoassay, and a detection zone on the solid support comprising immobilized anti-S100A9 antibody such that any S100A9 polypeptide in the sample forms an antibody-antigen-antibody sandwich with labeled anti-calprotectin antibody and with the immobilized anti-S100A9 antibody in the detection zone.

[0182] These aspects are described further below in terms of devices including the significant elements for performing such a lateral flow sandwich immunoassay.

[0183] Accordingly, another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(3) a conjugate zone having mobilizable labeled anti-calprotectin antibody located on the solid support; and

(4) a sample pad for application of a sample in operable contact with the first end of the solid support;

wherein a sample applied to the sample pad migrates to the conjugate zone to solubilize and mobilize the labeled anti-calprotectin antibody and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0184] The device can further include an optional control zone and a casing with apertures as described above. If the control zone is used, and the control zone includes an unrelated antigen, the device includes a labeled antibody specifically binding the unrelated antigen as described above.

[0185] Yet another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support; and

(3) a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the first end of the solid support;

wherein a sample applied to the conjugate pad solubilizes and mobilizes the labeled anti-calprotectin antibody in the conjugate pad and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the

immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0186] The device can further include an optional control zone and a casing with apertures as described above.

[0187] Still another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(3) a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the first end of the solid support; and

(4) a sample pad for application of the sample in operable contact with the conjugate pad;

wherein a sample applied to the sample pad migrates to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0188] Yet another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a conjugate pad having mobilizable labeled anti-calprotectin antibody; and

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-Si 00A9 antibody at the detection zone to perform a sandwich immunoassay.

[0189] Yet another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S1 00A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the sample pad; and

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the sample pad and the sample migrates to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S1 00A9 antibody at the detection zone to perform a sandwich immunoassay.

[0190] Still other alternatives employ a mobile labeled anti-calprotectin antibody, typically in solution as described above, that is added to the device with the sample and is not originally present in the device.

[0191] Accordingly, yet another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a solid support providing lateral flow having a first end and a second end;

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(3) a sample pad in operable contact with the first end of the solid support;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the sample pad, the sample and the mobile labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay

[0192] Accordingly, yet another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a solid support providing lateral flow having a first end and a second end; and

(2) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the solid support, the sample and the mobile labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0193] Yet another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a conjugate pad for

application of a mobile labeled anti-calprotectin antibody in operable contact with the sample pad; and

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the sample pad and a mobile labeled anti-calprotectin antibody is applied to the conjugate pad, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0194] Still another aspect of the invention is a device for performing a lateral flow sandwich immunoassay for S100A9 comprising:

(1) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(2) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a mobile labeled anti-calprotectin antibody to the sample pad;

(3) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the sample pad, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the sample pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

[0195] Still other devices can be constructed according to these general principles; modifications of these devices according to general principles known in the art. Such devices can include multiplex devices capable of assaying a plurality of

samples and/or standards in a single device. In general, such multiplex devices are constructed so that the multiple solid supports that provide lateral flow, one for each sample, are positioned in parallel so that the detection zones, and, if present, control zones, are at the same position relative to the end of the device for each sample and/or standard to be assayed.

[0196] Typically, the label of the mobilizable or mobile labeled anti-calprotectin antibody is a directly detectable label, such as a radioactive label, a fluorescent label, a chemiluminescent label, an electrochemiluminescent label, a bioluminescent label, a colloidal metal label such as a colloidal gold or colloidal silver label, a colloidal metal oxide label, a dye label, a colored latex particle label, a colored polystyrene or polypropylene particle label, a liposome label, a Surface-Enhanced Resonant Raman Spectroscopy (SERRS) label, or a signaling aptamer label. A preferred label is a colloidal metal label. A particularly preferred label is a colloidal gold or colloidal silver label. A more particularly preferred label is a colloidal gold label. Additional preferred labels are dye labels or colored latex particle labels.

[0197] As another alternative, an enzyme label such as those described above can be used. However, because the use of an enzyme label requires an additional incubation step, it is generally preferred to use a direct label such as those described above, especially a colloidal gold or colloidal silver label, a dye label, or a colored latex particle label.

[0198] Methods for immobilizing the anti-S1 00A9 antibody at the detection zone of the solid support that provides lateral flow are well known in the art. Immobilization can be either by covalent coupling to the solid support or non-covalent immobilization on the solid support. Suitable immobilization techniques are described, for example, in P. Tijssen, "Practice and Theory of Enzyme Immunoassays" (Elsevier, Amsterdam, 1985), ch. 13, pp. 297-328, incorporated herein by this reference.

[0199] Devices for reading direct labels such as colloidal gold or colloidal silver labels are known in the art. Such devices are described, for example, in R.C. Wong & H. Tse, eds, "Lateral Flow Immunoassay" (Humana Press, New York, NY, 2009).

[0200] Lateral flow assay devices for performing sandwich immunoassays for S100A9 polypeptide can be employed in the diagnosis and screening assays described above.

[0201] In one alternative, a method for determining the concentration of calprotectin in a sample employing a lateral flow immunoassay device for performing a sandwich immunoassay comprises the steps of:

(1) applying the sample to a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention;

(2) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody; and

(3) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone.

[0202] Similarly, another method employing a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention is a method of detecting or diagnosing the presence of active inflammatory bowel disease comprising the steps of:

(1) applying a sample that may contain calprotectin to a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention;

(2) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(3) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone; and

(4) determnining the presence or absence of active inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case active inflammatory bowel disease is present in the patient, or is less than 50 mg/kg, in which case active inflammatory bowel disease is absent in the patient.

[0203] Similarly, yet another method employing a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention is a method of determining whether a patient with inflammatory bowel disease who has been in remission is at risk of suffering a relapse comprising the steps of:

(1) applying a sample that may contain calprotectin to a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention;

(2) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to labeled anti-calprotectin antibody;

(3) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone; and

(4) determining the risk of relapse of inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease exists, or is less than 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease does not exist.

[0204] In these methods employing a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention, suitable labels for the labeled anti-calprotectin antibody and methods for determining the quantity of the label bound at the detection zone are as described above.

[0205] In these methods, the method can further comprise the steps of constructing a standard curve and determining the concentration of calprotectin in the

sample by comparison with the standard curve, as described above. The standard curve can be constructed using a plurality of concentrations of calprotectin, or using a plurality of concentrations of purified S100A9 polypeptide, as described above.

[0206] Yet another method according to the present invention employing a lateral flow device for performing a sandwich immunoassay for S100A9 polypeptide is a method of performing an immunoassay for S100A9 polypeptide and for a related antigen to determine whether inflammatory bowel disease exists in a subject, the method comprising the steps of:

(1) providing a stool sample from a subject;

(2) applying the sample to a lateral flow immunoassay device for performing a sandwich immunoassay according to the present invention;

(3) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(4) performing an immunoassay for a related antigen;

(5) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone;

(6) determining the concentration of the related antigen in the stool sample from the results of the immunoassay for the related antigen; and

(7) comparing the concentration of S100A9 polypeptide and the concentration of the related antigen in the stool sample with the cutoff values for S100A9 polypeptide and the related antigen to determine whether or not inflammatory bowel disease exists in the subject.

[0207] As described above, the cutoff value for calprotectin is 50 mg/kg.

[0208] As described above, the term "related antigen" means that the antigen in question is one that is associated with the occurrence of inflammatory bowel disease and can be used to discriminate between patients who have inflammatory bowel disease and patients who do not have inflammatory bowel disease.

[0209] In one alternative, the related antigen is S100A12. A suitable cutoff value for S100A12 is 66 pg/kg (T. Kaiser et al,. "Faecal S100A12 as a Non-invasive Marker Distinguishing Inflammatory Bowel Disease from Irritable Bowel Syndrome," Gut 56:1706-13 (2007)). Typically, if the related antigen is S100A12, the immunoassay is an ELISA sandwich immunoassay; the ELISA sandwich immunoassay for S100A12 can employ an unlabeled monoclonal anti-S100A12 antibody and an enzyme-labeled polyclonal anti-S100A12 antibody. However, other alternatives are possible for the unlabeled capture antibody and the labeled antibody in an ELISA immunoassay. Additionally, the immunoassay for the related antigen, such as S100A12, can be an immunoassay other than an ELISA immunoassay. Immunoassays are described in D. Wild, ed., "The Immunoassay Handbook" (3rd ed., Elsevier, Amsterdam, 2005).

[0210] In another alternative, the related antigen is lactoferrin. Immunoassays for lactoferrin are well known in the art and are described above. Methods for assay of lactoferrin as known in the art can include sandwich immunoassays such as ELISA immunoassays and lateral flow immunoassays, as well as competitive immunoassays. A suitable cutoff value for lactoferrin is 2.2 ng/mL.

[0211] The immunoassay for the related antigen can be performed by a competitive immunoassay method such as a competitive immunoassay on a solid phase platform. The immunoassay for the related antigen can also be another conventional competitive immunoassay, such as a homogeneous or heterogeneous competitive immunoassay, or a sandwich immunoassay such as a conventional ELISA or a conventional lateral flow immunoassay. Immunoassays are described in D.G. Wild, ed., "The Immunoassay Handbook" (3rd ed., Elsevier, Amsterdam, 2005).

[0212] Yet another aspect of the present invention is a kit for immunoassay of S100A9 and diagnosis, screening, or monitoring of inflammatory bowel disease comprising, separately packaged:

(1) a solid support for performing a lateral flow sandwich immunoassay for detection of S100A9 polypeptide; and

(2) a labeled anti-calprotectin antibody to be applied to the solid support.

[0213] The quantity of the labeled anti-calprotectin antibody can be such that the quantity is intended to be used in a single assay, in which case the kit includes a single solid support. Alternatively, the quantity can be such that it is intended to be used in a plurality of individual assays, such as 2, 3, 5, 10, 15, 20, 25, 30, 40, 50, or 100 individual assays, and the appropriate quantity for a single assay measured out by the individual performing the assay, in which case the kit includes a plurality of solid supports, and the quantity of labeled anti-calprotectin antibody is sufficient for a plurality of assays such that the quantity of labeled anti-calprotectin antibody is sufficient for use with each of the solid supports included in the kit.

[0214] In addition, a kit according to the present invention can further include one or more of the following items, all separately packaged:

(1) in the event that the label of the labeled anti-calprotectin antibody is an enzyme label, a substrate for the enzyme label; and

(2) a set of S100A9 polypeptide standards of differing concentrations so that a standard curve for S100A9 polypeptide can be established.

[0215] Additionally, although these assays have been described with particular attention to the use of calprotectin as a marker for inflammatory bowel disease, calprotectin can be used as a marker for the existence of inflammation in general. Specifically, calprotectin can be used as a marker for many inflammatory diseases and conditions, including, but not limited to, allograft rejection, coronary heart disease, atheromatosis, sepsis, preeclampsia, cirrhosis, periodontitis, obesity, type 2 diabetes, acne vulgaris, asthma, autoimmune disorders, chronic prostatitis, glomerulonephritis, pelvic inflammatory disease, sarcoidosis, and vasculitis. Because inflammatory processes associated with calprotectin contribute to the etiology of cancer, including pancreatic cancer, ovarian cancer, and uterine cancer, and are especially prominent in inflammatory breast cancer, assay of calprotectin concentration can also be used in the diagnosis and monitoring of cancer.

[0216] The invention is illustrated by the following Examples. These Examples are included for illustrative purposes only, and are not intended to limit the invention.

EXAMPLES

Example 1

ELISA Immunoassay for S100A8 (Comparative Example)

[0217] An ELISA immunoassay was performed employing unlabeled monoclonal antibodies specifically binding S100A8 polypeptide as the capture antibody and alkalkine-phosphate-labeled polyclonal antibody as the detection antibody. The standard curve, established using purified recombinant A8 polypeptide, is shown in Figure 9.

[0218] Thirteen stool extracts with calprotectin concentrations of between 21 and 817 ng/mL were tested using this ELISA immunoassay for A8, but they all gave a signal clearly below that of the lowest A8 standard. This supports the conclusion that antibodies specific for A8 should be avoided in immunoassays for calprotectin in cell extracts.

Example 2

ELISA Immunoassay for S100A9 Employing Calprotectin Standards with Monoclonal Capture Antibody and Polyclonal Enzyme-Labeled Detection Antibody

[0219] Coating of Microwells and Performance of the Immunoassay: 96 well microplates with high protein binding capacity, for instance MaxiSorp™ from Nunc, can be used. For coating, to each well is added 100 to 200 µl, preferably 150 µl, of a highly selected Mab, for instance the Calpro Mab CAL1-4H1/2/2, in a suitable concentration, for instance 1 to 4 pg/ml, preferably 2 pg/ml, in 0.1 M sodium citrate, pH 6. The wells are covered by vapor tight adhesive plastic and stored at +4° C for a suitable period of time, for instance six hours to several weeks, preferably 18 hours. Before use, excess antibodies are removed by washing each well three to four times with a suitable

washing buffer. To different wells are added 50 µl to 150 µl, preferably 50 µl, of standards with known concentrations of calprotectin or A9 and samples in a suitable buffer 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 50 µl, of an enzyme-conjugated highly selected immunoaffinity purified polyclonal antibody in a suitable buffer dilution, 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 p-nitrophenylphosphate, is added to each well. The plate is left at room temperature for 10 to 60 minutes, preferably 30 minutes, after which the color intensity in each well is measure by an ELISA reader. The concentration of calprotectin in the samples is determined by comparison of the color intensities in the respective wells with those of the standards taking the sample dilution factor into the calculation.

[0220] Results: Figure 10 shows a typical standard curve from such an ELISA (i.e., an ELISA employing unlabeled monoclonal anti-S100A9 antibody as the capture antibody and alkaline-phosphatase-labeled polyclonal anti-S100A9 as the detection antibody).

Example 3

ELISA Immunoassay for S100A9 Employing Calprotectin Standards with Monoclonal Capture Antibody and Monoclonal Detection Antibody

[0221] A similar ELISA immunoassay for S100A9 was performed using the same monoclonal capture anti-S100A9 antibody employed in the ELISA immunoassay of Example 2 and the same monoclonal anti-S100A9 antibody labeled with the enzyme alkaline phosphatase as the detection antibody. Except for the substitution of the enzyme-labeled monoclonal antibody for the enzyme-labeled polyclonal antibody of

Example 2, the immunoassay was performed exactly the same way as the immunoassay of Example 2.

[0222] The results are shown in Figure 11.

Example 4

ELISA Immunoassay for S100A9 Employing A9 Standards with Monoclonal Capture Antibody and Polyclonal Enzyme-Labeled Detection Antibody

[0223] An ELISA immunoassay for S100A9 was performed employing a monoclonal anti-S100A9 capture antibody and an enzyme-labeled polyclonal anti-S100A9 detection antibody, as described in Example 2, using purified S100A9 protein as the standard.

[0224] The correlation between the results is shown in Figure 12. Figure 12 is a graph showing the correlation between a standard curve for an A9 ELISA using unlabeled monoclonal anti-S100A9 antibody as the capture antibody and alkaline-phosphatase-labeled polyclonal anti-S100A9 as the detection antibody, employing recombinant calprotectin as the standard, and a standard curve for an A9 ELISA using unlabeled monoclonal anti-S100A9 antibody as the capture antibody and alkaline-phosphatase-labeled polyclonal anti-S100A9 as the detection antibody, employing purified recombinant S100A9 as the standard. There was almost an exact correlation; r^2 was 0.9936. These results indicate that either calprotectin itself or its S100A9 subunit can be used as the standard in immunoassays for calprotectin without loss of accuracy.

Example 5

Comparison Between Results of Example 2 and Example 3

[0225] A comparison between the results of Example 2 (using a monoclonal anti-S100A9 capture antibody and a polyclonal enzyme-labeled anti-S100A9 detection

antibody) and Example 3 (using a monoclonal anti-S100A9 capture antibody and the same antibody as enzyme-labeled detection antibody) is shown in Table 1, below. In Table 1, "M/P" refers to Example 2 (with a monoclonal capture antibody and a polyclonal detection antibody), and "M/M" refers to Example 3 (with a monoclonal capture antibody and a monoclonal detection antibody). Results are presented in terms of ng/mL.

Extract	M/P	M/M
1	376	263
2	315	180
3	421	233
4	357	301
5	145	81
6	315	178
7	236	151
8	380	263
9	481	301
10	375	178

[0226] Clearly, the new ELISA using a combination of monoclonal and polyclonal antibodies gave higher values. The discrepancies were even higher for samples with concentrations above 500 ng/ml. 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 monoclonals reacted with A9 epitopes, some of them were probably less available or absent on calprotectin in the extracts.

[0227] Therefore, in most cases, it is generally preferred to use a combination of monoclonal anti-S100A9 capture antibody and polyclonal enzyme-labeled anti-

calprotectin detection antibody in an ELISA sandwich immunoassay for calprotectin, particularly on heterogeneous samples such as stool extracts containing extraneous and potentially interfering components. The combination yields better reactivity if such components are present. However, if the combination of combination of monoclonal anti-S100A9 capture antibody and polyclonal enzyme-labeled anti-calprotectin detection antibody is used, either purified calprotectin or purified S100A9 protein (e.g., recombinant S100A9 protein) can be used as a standard.

ADVANTAGES OF THE INVENTION

[0228] The present invention provides an efficient, accurate, and reproducible immunoassay for calprotectin, and thus an improved bioassay for a marker that is useful for the detection, diagnosis, or monitoring of inflammatory bowel disease. The immunoassay is particularly useful for assay of calprotectin in stool samples and is reproducible and less subject to interference from components of stool samples than existing assays. The immunoassay also avoids falsely low values when stool samples with high concentrations of calprotectin are tested, which is a problem that frequently occurs with existing assays.

[0229] Methods and kits according to the present invention possess industrial applicability for the *in vitro* assay of calprotectin.

[0230] The inventions illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the future shown and described or any portion thereof, and it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and

variation of the inventions herein disclosed can be resorted by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the scope of the generic disclosure also form part of these inventions. This includes the generic description of each invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised materials specifically resided therein.

[0231] In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. It is also to be understood that the above description is intended to be illustrative and not restrictive. Many embodiments will be apparent to those of in the art upon reviewing the above description. The scope of the invention should therefore, be determined not with reference to the above description, but should instead be determined with reference to the appended claims, along with the full scope of equivalents to which such claims are entitled. The disclosures of all articles and references, including patent publications, are incorporated herein by reference. When claims are written in a form employing the transitional phrase "comprising," the recitation of "comprising" also encompasses any and all embodiments described by claims in which the transitional phrase is "consisting essentially of or "consisting of," and, should it prove advantageous to do so, the transitional phrases "consisting essentially of" or "consisting of" could be used in place of "comprising."

What is claimed is:

1. A method for determining the concentration of calprotectin in a sample comprising the steps of:

(a) providing a monoclonal anti-S100A9 antibody;

(b) coating the monoclonal anti-S100A9 antibody onto a solid support;

(c) reacting the solid support with coated monoclonal anti-S1 00A9 antibody with a sample that may contain calprotectin to bind any calprotectin in the sample to the solid support;

(d) washing the solid support;

(e) reacting the solid support with a labeled polyclonal anti-calprotectin antibody to bind the labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support, the labeled polyclonal anti-calprotectin antibody being able to produce a detectable signal proportional to the concentration of the antibody bound to the calprotectin bound to the solid support; and

(f) measuring the detectable signal to determine the concentration of calprotectin in the sample.

2. The method of claim 1 wherein the monoclonal antibody to A9 is produced using a recombinant A9 protein as immunogen.

3. The method of claim 2 wherein the recombinant A9 protein has the sequence

MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVI
EHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP
(SEQ ID NO: 1).

4. The method of claim 3 wherein the recombinant A9 protein of SEQ ID NO: 1 is produced by expression of a DNA molecule with the sequence

GGTACCATATGACCTGCAAAATGAGCCAGCTGGAACGTAACATTGAAACCATCATC
AACACCTTTCATCAGTATAGCGTGAAACTGGGCCATCCGGATACCCTGAACCAGG
GCGAATTTATGATCGAACACATCATGGAAGATCTGGATACCAACGCGGATAAACAG
CTGTCTTTCGAAGAAGAACTGGTGCGTAAAGATCTGCAGAACTTCCTGAAAAAAGA
AAACAAAAACGAAAAAGAATTTATTATGCTGATGGCGCGTCTGACCTGGGCGAGCC

ATGAAAAAATGCATGAAGGCGATGAAGGCCCGGGTCATCATCATAAACCGGGCCT GGGCGAAGGCACCCCGTGATAACTCG (SEQ ID NO: 3).

5. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A8.

6. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A9.

7. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding a conformational epitope incorporating both S100A8 and S100A9.

8. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with all of calprotectin, isolated S100A8, and isolated S100A9.

9. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A8 but not at all or to a significantly lesser degree with isolated S100A9.

10. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A9 but not at all or to a significantly lesser degree with isolated S100A8.

11. The method of claim 1 wherein the labeled polyclonal anti-calprotectin antibody is an enzyme-labeled antibody, and wherein the method comprises the step of incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce the detectable signal.

12. The method of claim 11 wherein the enzyme produces a product that is detected and/or quantitated photometrically.

13. The method of claim 11 wherein the enzyme produces a product that is detected and/or quantitated by a technique selected from the group consisting of detection and/or quantitation of fluorescence, detection and/or quantitation of chemiluminescence, and detection and/or quantitation of bioluminescence.

14. The method of claim 11 wherein the enzyme of the enzyme-labeled antibody is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase.

15. The method of claim 14 wherein the enzyme of the enzyme-labeled antibody is alkaline phosphatase.

16. The method of claim 1 wherein the buffer used to coat the solid support with the monoclonal antibody is a citrate buffer.

17. The method of claim 16 wherein the citrate buffer is at a concentration of from about 50 mM to about 150 mM.

18. The method of claim 16 wherein the pH of the citrate buffer is from about pH 5 to about pH 7.

19. The method of claim 16 wherein the citrate buffer is 0.1 M sodium citrate, pH 6.0.

20. The method of claim 1 wherein the solid support is constructed of a synthetic resin selected from the group consisting of polystyrene, Luran, polypropylene, and polyvinyl chloride.

21. The method of claim 1 wherein the solid support is in the form of a microwell plate with 96 wells.

22. The method of claim 1 wherein the monoclonal antibody is used to coat the solid support at a concentration of from about 1 to 4 μg/mL.

23. The method of claim 1 wherein, during the process of coating the solid support, the solid support is stored at a temperature of from about 0° C to about 8° C.

24. The method of claim 1 wherein during the process of coating the solid support, the solid support is stored at a temperature of from about 9° C to about 37° C.

25. The method of claim 1 wherein, during the process of coating the solid support, the solid support is stored for an incubation period of from about 6 hours to several weeks.

26. The method of claim 1 wherein the size of the sample is from about 50 µl to about 150 µl per assay or standard to be performed.

27. The method of claim 1 further comprising the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

28. The method of claim 27 wherein the standard curve is constructed using a plurality of concentrations of purified calprotectin.

29. The method of claim 27 wherein the standard curve is constructed using a plurality of concentrations of purified A9 polypeptide.

30. The method of claim 29 wherein the purified A9 polypeptide is a cloned recombinant A9 polypeptide.

31. The method of claim 1 wherein, subsequent to the addition of sample to the solid support, the sample and solid support are incubated at a temperature of from about 20° C to about 25° C from about 10 minutes to about 60 minutes.

32. The method of claim 31 wherein the sample and solid support are incubated about 40 minutes.

33. The method of claim 1, wherein, subsequent to the addition of sample to the solid support and subsequent to the incubation of the sample and solid support and a washing step, from about 50 µl to about 150 µl of a labeled highly immunoaffinity purified polyclonal anti-calprotectin antibody is added to each assay or standard to be performed.

34. The method of claim 33 wherein the labeled highly immunoaffinity purified polyclonal anti-S100A9 antibody is an enzyme-conjugated highly immunoaffinity purified polyclonal anti-S100A9 antibody.

35. The method of claim 11 wherein, subsequent to incubation of the solid support with the enzyme-labeled antibody and a washing step, from about 50 µl to about 200 µl of a substrate for the enzyme of the enzyme-labeled antibody is added to each assay or standard to be performed.

36. The method of claim 35 wherein the enzyme is alkaline phosphatase and the substrate is p-nitrophenylphosphate.

37. The method of claim 11 wherein the solid support is incubated for about 10 minutes to about 60 minutes subsequent to addition of a substrate for the enzyme.

38. The method of claim 1 wherein the sample is selected from the group consisting of a stool sample, a gastrointestinal tract sample, a whole blood sample, a serum sample, a plasma sample, a urine sample, and a crevicular fluid sample.

39. The method of claim 38 wherein the sample is a stool sample or a gastrointestinal tract sample.

40. The method of claim 39 wherein the sample is extracted prior to performance of the assay by a method comprising the steps of:

(i) mixing a small amount of sample with an excess amount of aqueous extraction buffer comprising at least one dissociating, disaggregating, and/or chelating agent;

(ii) homogenizing the sample in a closed tube; and

(iii) recovering the liquid extract resulting from the separation, which contains calprotectin as well as other proteins.

41. The method of claim 40 wherein the extraction buffer is a Tris buffer of pH 8 with added sodium citrate.

42. The method of claim 40 wherein the extraction buffer includes a dissociating, disaggregating, and/or chelating agent selected from the group consisting of sodium dodecyl sulfate and urea.

43. The method of claim 40 wherein the extraction buffer includes from about 0.5% to about 2% of bovine serum albumin.

44. A method for diagnosing or detecting the presence of active inflammatory bowel disease comprising the steps of:

(a) providing a monoclonal anti-S100A9 antibody;

(b) coating the monoclonal anti-S100A9 antibody onto a solid support;

(c) reacting the solid support with coated monoclonal anti-S1 00A9 antibody with a stool sample from a patient suspected of having inflammatory bowel disease to bind any calprotectin in the sample to the solid support;

(d) washing the solid support;

(e) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-S100A9 antibody to the calprotectin bound to the solid support;

(f) incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce a detectable signal;

(g) measuring the detectable signal to determine the concentration of calprotectin in the sample; and

(h) determining the presence or absence of active inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case active inflammatory bowel disease is present in the patient, or is less than 50 mg/kg, in which case active inflammatory bowel disease is absent in the patient.

45. The method of claim 44 wherein the monoclonal antibody to A9 is produced using a recombinant A9 protein as immunogen.

46. The method of claim 45 wherein the recombinant A9 protein has the sequence
MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVI
EHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP
(SEQ ID NO: 1).

47. The method of claim 46 wherein the recombinant A9 protein of SEQ ID NO: 1 is produced by expression of a DNA molecule with the sequence
GGTACCATATGACCTGCAAAATGAGCCAGCTGGAACGTAACATTGAAACCATCATC
AACACCTTTCATCAGTATAGCGTGAAACTGGGCCATCCGGATACCCTGAACCAGG
GCGAATTTATGATCGAACACATCATGGAAGATCTGGATACCAACGCGGATAAACAG
CTGTCTTTCGAAGAAGAACTGGTGCGTAAAGATCTGCAGAACTTCCTGAAAAAAGA
AAACAAAAACGAAAAAGAATTTATTATGCTGATGGCGCGTCTGACCTGGGCGAGCC

ATGAAAAAATGCATGAAGGCGATGAAGGCCCGGGTCATCATCATAAACCGGGCCT GGGCGAAGGCACCCCGTGATAACTCG (SEQ ID NO: 3).

48. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A8.

49. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A9.

50. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding a conformational epitope incorporating both S100A8 and S100A9.

51. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with all of calprotectin, isolated S100A8, and isolated S100A9.

52. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A8 but not at all or to a significantly lesser degree with isolated S100A9.

53. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A9 but not at all or to a significantly lesser degree with isolated S100A8

54. The method of claim 44 wherein the labeled polyclonal anti-calprotectin antibody is an enzyme-labeled antibody, and wherein the method comprises the step of incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce the detectable signal.

55. The method of claim 54 wherein the enzyme produces a product that is detected and/or quantitated photometrically.

56. The method of claim 54 wherein the enzyme produces a product that is detected and/or quantitated by a technique selected from the group consisting of detection and/or quantitation of fluorescence, detection and/or quantitation of chemiluminescence, and detection and/or quantitation of bioluminescence.

57. The method of claim 54 wherein the enzyme of the enzyme-labeled antibody is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase.

58. The method of claim 57 wherein the enzyme of the enzyme-labeled antibody is alkaline phosphatase.

59. The method of claim 44 wherein the buffer used to coat the solid support with the monoclonal antibody is a citrate buffer.

60. The method of claim 59 wherein the citrate buffer is at a concentration of from about 50 mM to about 150 mM.

61. The method of claim 59 wherein the pH of the citrate buffer is from about pH 5 to about pH 7.

62. The method of claim 59 wherein the citrate buffer is 0.1 M sodium citrate, pH 6.0.

63. The method of claim 44 further comprising the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

64. The method of claim 63 wherein the standard curve is constructed using a plurality of concentrations of purified calprotectin.

65. The method of claim 63 wherein the standard curve is constructed using a plurality of concentrations of purified S100A9 polypeptide.

66. The method of claim 65 wherein the purified S100A9 polypeptide is a cloned recombinant S100A9 polypeptide.

67. A method for determining whether a patient with inflammatory bowel disease who has been in remission is at risk of suffering a relapse comprising the steps of:

(a) providing a monoclonal anti-S100A9 antibody;

(b) coating the monoclonal anti-S100A9 antibody onto a solid support;

(c) reacting the solid support with coated monoclonal anti-S100A9 antibody with a stool sample from a patient with inflammatory bowel disease that is in remission to bind any calprotectin in the sample to the solid support;

(d) washing the solid support;

(e) reacting the solid support with an enzyme-labeled polyclonal anti-calprotectin antibody to bind the enzyme-labeled polyclonal anti-calprotectin antibody to the calprotectin bound to the solid support;

(f) incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce a detectable signal;

(g) measuring the detectable signal to determine the concentration of calprotectin in the sample; and

(h) determining the risk of relapse of inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease exists, or is less than 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease does not exist.

68. The method of claim 67 wherein the monoclonal antibody to S100A9 is produced using a recombinant S100A9 protein as immunogen.

69. The method of claim 68 wherein the recombinant S100A9 protein has the sequence
MTCKMSQLERNIETIINTFHQYSVKLGHPDTLNQGEFKELVRKDLQNFLKKENKNEKVI
EHIMEDLDTNADKQLSFEEFIMLMARLTWASHEKMHEGDEGPGHHHKPGLGEGTP
(SEQ ID NO: 1).

70. The method of claim 69 wherein the recombinant S100A9 protein of SEQ ID NO: 1 is produced by expression of a DNA molecule with the sequence
GGTACCATATGACCTGCAAAATGAGCCAGCTGGAACGTAACATTGAAACCATCATC
AACACCTTTCATCAGTATAGCGTGAAACTGGGCCATCCGGATACCCTGAACCAGG
GCGAATTTATGATCGAACACATCATGGAAGATCTGGATACCAACGCGGATAAACAG
CTGTCTTTCGAAGAAGAACTGGTGCGTAAAGATCTGCAGAACTTCCTGAAAAAAGA
AAACAAAAACGAAAAAGAATTTATTATGCTGATGGCGCGTCTGACCTGGGCGAGCC
ATGAAAAAATGCATGAAGGCGATGAAGGCCCGGGTCATCATCATAAACCGGGCCT
GGGCGAAGGCACCCCGTGATAACTCG (SEQ ID NO: 3).

71. The method of claim 67 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A8.

72. The method of claim 67 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A9.

73. The method of claim 67 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding a conformational epitope incorporating both S100A8 and S100A9.

74. The method of claim 67 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with all of calprotectin, isolated S100A8, and isolated S100A9.

75. The method of claim 67 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A8 but not at all or to a significantly lesser degree with isolated S100A9.

76. The method of claim 67 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A9 but not at all or to a significantly lesser degree with isolated S100A8.

77. The method of claim 67 wherein the labeled polyclonal anti-S100A9 antibody is an enzyme-labeled antibody, and wherein the method comprises the step of incubating the solid support with a substrate for the enzyme of the enzyme-labeled polyclonal antibody to produce the detectable signal.

78. The method of claim 77 wherein the enzyme produces a product that is detected and/or quantitated photometrically.

79. The method of claim 77 wherein the enzyme produces a product that is detected and/or quantitated by a technique selected from the group consisting of detection and/or quantitation of fluorescence, detection and/or quantitation of chemiluminescence, and detection and/or quantitation of bioluminescence.

80. The method of claim 77 wherein the enzyme of the enzyme-labeled antibody is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase.

81. The method of claim 80 wherein the enzyme of the enzyme-labeled antibody is alkaline phosphatase.

82. The method of claim 67 wherein the buffer used to coat the solid support with the monoclonal antibody is a citrate buffer.

83. The method of claim 82 wherein the citrate buffer is at a concentration of from about 50 mM to about 150 mM.

84. The method of claim 82 wherein the pH of the citrate buffer is from about pH 5 to about pH 7.

85. The method of claim 82 wherein the citrate buffer is 0.1 M sodium citrate, pH 6.0.

86. The method of claim 67 further comprising the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

87. The method of claim 86 wherein the standard curve is constructed using a plurality of concentrations of purified calprotectin.

88. The method of claim 86 wherein the standard curve is constructed using a plurality of concentrations of purified S100A9 polypeptide.

89. The method of claim 88 wherein the purified A9 polypeptide is a cloned recombinant S100A9 polypeptide.

90. A method of selecting a polyclonal or monoclonal anti-S100A9 antibody for high binding affinity to S100A9 polypeptide comprising the steps of:

(a) providing a plurality of polyclonal or monoclonal antibodies binding S100A9 protein;

(b) separately reacting each of the polyclonal or monoclonal antibodies with recombinant S100A9 protein under conditions where equilibrium is reached;

(c) determining the affinity of each of the polyclonal or monoclonal antibodies for S100A9 polypeptide; and

(d) selecting the polyclonal or monoclonal antibody with the highest affinity for S100A9 polypeptide.

91. The method of claim 90 wherein the antibodies provided for selection are polyclonal antibodies.

92. The method of claim 90 wherein the antibodies provided for selection are monoclonal antibodies.

93. The method of claim 90 further comprising the step of comparing the affinity of the antibody selected in step (d) with a standard antibody.

94. The method of claim 93 wherein the standard antibody is Calpro Mab CAL1-4H1/2/2.

95. The method of claim 90 wherein the antibody selected in step (d) has an affinity of at least 80% of the affinity of Calpro Mab CAL1-4H1/2/2.

96. A method of selecting a polyclonal or monoclonal anti-S100A9 antibody for low cross-reactivity to a polypeptide selected from the group consisting of S100A8 polypeptide and S100A12 polypeptide comprising the steps of:

(a) providing a polyclonal or monoclonal antibody binding S100A9 protein;

(b) reacting the polyclonal or monoclonal antibody with recombinant S100A9 protein under conditions where equilibrium is reached; and

(c) determining the affinity of the polyclonal or monoclonal antibody for S100A9 protein;

(d) reacting the polyclonal or monoclonal antibody with a recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein under conditions where equilibrium is reached;

(e) determining the affinity of the polyclonal or monoclonal antibody for the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein;

(f) comparing the affinity of the polyclonal or monoclonal antibody for recombinant S100A9 protein and for the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein to

determine the cross-reactivity of the polyclonal or monoclonal antibody with the recombinant protein selected from the group consisting of recombinant A8 protein and recombinant S100A12 protein; and

(g) selecting the polyclonal or monoclonal antibody with the lowest cross-reactivity with the recombinant protein selected from the group consisting of recombinant S100A8 protein and recombinant S100A12 protein.

97. The method of claim 96 wherein the antibodies provided for selection are polyclonal antibodies.

98. The method of claim 96 wherein the antibodies provided for selection are monoclonal antibodies.

99. The method of claim 96 wherein the polypeptide selected from the group consisting of A8 polypeptide and A12 polypeptide is A8 polypeptide.

100. The method of claim 96 wherein the polypeptide selected from the group consisting of A8 polypeptide and A12 polypeptide is A12 polypeptide.

101. The method of claim 96 wherein the antibody selected in step (g) has a cross-reactivity with the recombinant protein selected from the group consisting of recombinant A8 protein and recombinant A12 protein of no greater than about 0.10.

102. A method of immunoaffinity purification of polyclonal anti-calprotectin antibodies to remove antibodies against epitopes present on standards but not on calprotectin in actual stool samples comprising the steps of:

(a) providing a polyclonal anti-S100A9 antibody;

(b) performing immunoaffinity purification with an affinity ligand selected from the group consisting of a recombinant calprotectin subunit and a related S100 protein so that antibody molecules binding the affinity ligand are retained by the affinity ligand; and

(c) isolating a fraction of polyclonal antibody not retained by the affinity ligand in order to remove antibodies against epitopes present on standards but not on calprotectin in actual stool samples.

103. The method of claim 102 wherein the affinity ligand is the S100A8 subunit.

104. The method of claim 102 wherein the affinity ligand is the S100A12 subunit.

105. The method of claim 102 wherein the affinity ligand is a S100 protein selected from the group consisting of S100A1 protein, the S100A2 protein, the S100A3 protein, the S100A4 protein, the S100A5 protein, the S100A6 protein, the S100A7 protein, the S100A10 protein, the S100A11 protein, the S100A12 protein, the S100A13 protein, the S100A14 protein, the S100A15 protein, and the S100A16 protein.

106. A method for the selection and removal of anti-calprotectin antibodies cross-reacting with another S100 protein other than S100A8 or S100A9 comprising the steps of:

(a) providing a polyclonal anti-S100A9 antibody;

(b) performing immunoaffinity purification with at least one affinity ligand that is a S100 protein other than S100A8 or S100A9; and

(c) isolating a fraction of polyclonal antibody not retained by the affinity ligand in order to remove anti-calprotectin antibodies that cross-react with at least one S100 protein other than S100A8 or S100A9.

107. The method of claim 106 wherein the step of performing affinity purification is performed employing multiple ligands in a single affinity column.

108. The method of claim 107 wherein the multiple ligands include at least two ligands selected from the group consisting of the S100A1 protein, the S100A2 protein, the S100A3 protein, the S100A4 protein, the S100A5 protein, the S100A6 protein, the S100A7 protein, the S100A10 protein, the S100A11 protein, the S100A12 protein, the S100A13 protein, the S100A14 protein, the S100A15 protein, and the S100A16 protein.

109. The method of claim 106 wherein the step of performing affinity purification is performed employing multiple ligands wherein each ligand is bound to an individual affinity column, and multiple affinity columns are employed.

110. The method of claim 109 wherein the multiple ligands include at least two ligands selected from the group consisting of the S100A1 protein, the S100A2 protein, the S100A3 protein, the S100A4 protein, the S100A5 protein, the S100A6

protein, the S100A7 protein, the S100A10 protein, the S100A11 protein, the S100A12 protein, the S100A13 protein, the S100A14 protein, the S100A15 protein, and the S100A16 protein.

111. A method for the selection of anti-S100A9 antibody preparations that give correct readings and provide a wide assay range in an ELISA comprising the steps of:

(a) providing an unlabeled monoclonal anti-S100A9 antibody and an enzyme-labeled polyclonal anti-S100A9 antibody that have been shown to give accurate results in an ELISA assay with stool samples;

(b) providing a candidate preparation of unlabeled monoclonal anti-Si 00A9 antibody;

(c) providing a candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody;

(d) performing an ELISA sandwich immunoassay using the unlabeled monoclonal anti-S100A9 antibody and the enzyme-labeled polyclonal anti-S100A9 antibody of (a) using a plurality of stool samples;

(e) performing an ELISA sandwich immunoassay using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody;

(f) comparing the results from the ELISA sandwich immunoassays of steps (d) and (e) to determine whether the results are substantially equivalent; and

(g) selecting candidate preparations of unlabeled monoclonal anti-Si 00A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that produce substantially equivalent results on the plurality of stool samples, such that results obtained using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody differ from the results obtained using a preparation of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-S100A9 antibody that have been shown to give accurate results on stool samples vary by no more than about 10% for any stool sample tested.

112. The method of claim 111 wherein the results obtained using the candidate preparation of unlabeled monoclonal anti-S100A9 antibody and the candidate preparation of enzyme-labeled polyclonal anti-S100A9 antibody differ from the results obtained using a preparation of unlabeled monoclonal anti-S100A9 antibody and enzyme-labeled polyclonal anti-calprotectin antibody that have been shown to give accurate results on stool samples vary by no more than about 5% for any stool sample tested.

113. A method of performing an immunoassay for S100A9 and for a related antigen to determine whether inflammatory bowel disease exists in a subject, the method comprising the steps of:

(a) providing a stool sample from a subject;

(b) performing an ELISA sandwich immunoassay for S100A9 using an unlabeled monoclonal anti-S100A9 antibody and an enzyme-labeled polyclonal anti-calprotectin antibody;

(c) performing an immunoassay for a related antigen;

(d) determining the concentration of calprotectin in the stool sample from the results of the ELISA sandwich immunoassay for S100A9;

(e) determining the concentration of the related antigen in the stool sample from the results of the immunoassay for the related antigen; and

(f) comparing the concentration of S100A9 and the concentration of the related antigen in the stool sample with the cutoff values for S100A9 and the related antigen to determine whether or not inflammatory bowel disease exists in the subject.

114. The method of claim 113 wherein the related antigen is S100A12.

115. The method of claim 113 wherein the related antigen is lactoferrin.

116. A kit for immunoassay of S100A9 and diagnosis, screening, or monitoring of inflammatory bowel disease comprising, separately packaged:

(a) a quantity of an unlabeled monoclonal anti-S100A9 antibody suitable for performance of an ELISA sandwich immunoassay for the detection of S100A9; and

(b) a quantity of an enzyme-labeled polyclonal anti-calprotectin antibody suitable for performance of an ELISA sandwich immunoassay for the detection of S100A9.

117. The kit of claim 116 wherein the kit includes a quantity of an unlabeled monoclonal anti-S100A9 antibody and a quantity of a labeled polyclonal anti-calprotectin antibody each sufficient for a single assay.

118. The kit of claim 116 wherein the kit includes a quantity of an unlabeled monoclonal anti-S100A9 antibody and a quantity of a labeled polyclonal anti-calprotectin antibody sufficient for a plurality of assays.

119. The kit of claim 116 wherein the kit further includes at least one of:

(c) a solid support suitable for performance of the ELISA immunoassay;

(d) wash buffer for washing the solid support after coating the solid support with immobilized antibody and after applying the sample to the solid support;

(e) substrate for the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody; and

(f) a set of S100A9 standards of differing concentrations so that a standard curve for S100A9 can be established.

120. The kit of claim 119 wherein the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody is selected from the group consisting of alkaline phosphatase, horseradish peroxidase, glucose 6-phosphate dehydrogenase, and β-galactosidase.

121. The kit of claim 120 wherein the enzyme of the enzyme-labeled polyclonal anti-calprotectin antibody is alkaline phosphatase.

122. The kit of claim 121 wherein the kit further includes p-nitrophenylphosphate as a substrate for the alkaline phosphatase.

123. A device for performing a lateral flow sandwich immunoassay for S100A9 polypeptide comprising:

(a) a solid support for lateral flow;

(b) a labeled anti-calprotectin antibody that is either present on the device before the commencement of the immunoassay or is added to the device at the commencement of the immunoassay; and

(c) a detection zone on the solid support comprising immobilized anti-Si 00A9 antibody such that any S100A9 polypeptide in the sample forms an antibody-antigen-antibody sandwich with labeled anti-calprotectin antibody and with the immobilized anti-S100A9 antibody in the detection zone.

124. The device of claim 123 wherein the solid support is constructed of a material selected from the group consisting of nitrocellulose, glass fiber, paper, nylon, and a synthetic nanoporous polymer.

125. The device of claim 124 wherein the solid support is nitrocellulose.

126. The device of claim 123 wherein the labeled anti-calprotectin antibody is present on the device before the commencement of the immunoassay.

127. The device of claim 123 wherein the labeled anti-calprotectin antibody is added to the device at the commencement of the immunoassay.

128. The device of claim 123 further comprising a control zone.

129. The device of claim 128 wherein the control zone comprises an immobilized antibody capable of specifically binding the labeled anti-calprotectin antibody by binding to a portion of the antibody distinct from the paratope of the labeled anti-calprotectin antibody.

130. The device of claim 128 wherein the control zone comprises an immobilized unrelated antigen and the device comprises a labeled antibody specifically binding the immobilized unrelated antigen.

131. The device of claim 123 further comprising a casing having at least one aperture therein.

132. The device of claim 123 wherein the device comprises:

(a) a solid support providing lateral flow having a first end and a second end;

(b) a detection zone having immobilized anti-S1 00A9 antibody, the detection zone located on the solid support;

(c) a conjugate zone having mobilizable labeled anti-calprotectin antibody located on the solid support; and

(d) a sample pad for application of a sample in operable contact with the first end of the solid support;

wherein a sample applied to the sample pad migrates to the conjugate zone to solubilize and mobilize the labeled anti-calprotectin antibody and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

133. The device of claim 120 wherein the device comprises:

(a) a solid support providing lateral flow having a first end and a second end;

(b) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support; and

(c) a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the first end of the solid support;

wherein a sample applied to the conjugate pad solubilizes and mobilizes the labeled anti-calprotectin antibody in the conjugate pad and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

134. The device of claim 123 wherein the device comprises:

(a) a solid support providing lateral flow having a first end and a second end;

(b) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(c) a conjugate pad having mobilizable labeled anti-calprotectin antibody in operable contact with the first end of the solid support; and

(d) a sample pad for application of the sample in operable contact with the conjugate pad;

wherein a sample applied to the sample pad migrates to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody and the sample and the mobilized labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

135. The device of claim 123 wherein the device comprises:

(a) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(b) a second substantially planar part, the second substantially planar part comprising a conjugate pad having mobilizable labeled anti-calprotectin antibody; and

(c) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-Si 00A9 antibody at the detection zone to perform a sandwich immunoassay.

136. The device of claim 123 wherein the device comprises:

(a) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(b) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a conjugate pad having

mobilizable labeled anti-calprotectin antibody in operable contact with the sample pad; and

(c) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the sample pad and the sample migrates to the conjugate pad to solubilize and mobilize the labeled anti-calprotectin antibody, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the conjugate pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

137. The device of claim 123 wherein the device comprises:

(a) a solid support providing lateral flow having a first end and a second end;

(b) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

(c) a sample pad in operable contact with the first end of the solid support;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the sample pad, the sample and the mobile labeled anti-calprotectin antibody migrate to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

138. The device of claim 123 wherein the device comprises:

(a) a solid support providing lateral flow having a first end and a second end; and

(b) a detection zone having immobilized anti-S100A9 antibody, the detection zone located on the solid support;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the solid support, the sample and the mobile labeled anti-calprotectin antibody migrate

to the detection zone, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

139. The device of claim 123 wherein the device comprises:

(a) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(b) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a conjugate application pad for application of a mobile labeled anti-calprotectin antibody in operable contact with the sample pad; and

(c) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample is applied to the sample pad and a mobile labeled anti-calprotectin antibody is applied to the conjugate pad, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the sample pad and conjugate pad are placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

140. The device of claim 123 wherein the device comprises:

(a) a first substantially planar part, the first substantially planar part comprising a solid support that provides lateral flow, the solid support that provides lateral flow comprising a detection zone having immobilized anti-S100A9 antibody;

(b) a second substantially planar part, the second substantially planar part comprising a sample pad for application of a sample and a mobile labeled anti-calprotectin antibody to the sample pad;

(c) a hinge connecting the first substantially planar part with the second substantially planar part;

wherein, after a sample and a mobile labeled anti-calprotectin antibody are applied to the sample pad, the hinge connecting the first substantially planar part with the second substantially planar part is closed so that the sample pad is placed into operable contact with the solid support, such that any S100A9 polypeptide in the sample binds to the labeled anti-calprotectin antibody and to the immobilized anti-S100A9 antibody at the detection zone to perform a sandwich immunoassay.

141. The device of claim 123 wherein the label of the labeled anti-calprotectin antibody is a directly detectable label.

142. The device of claim 141 wherein the directly detectable label is selected from the group consisting of a radioactive label, a fluorescent label, a chemiluminescent label, an electrochemiluminescent label, a bioluminescent label, a colloidal metal label, a colloidal metal oxide label, a dye label, a colored latex particle label, a colored polystyrene or polypropylene particle label, a liposome label, a Surface-Enhanced Resonant Raman Spectroscopy (SERRS) label, and a signaling aptamer label.

143. The device of claim 142 wherein the directly detectable label is a colloidal metal label.

144. The device of claim 123 wherein the immobilized anti-S100A9 antibody is covalently immobilized at the detection zone.

145. The device of claim 123 wherein the immobilized anti-S100A9 antibody is non-covalently immobilized at the detection zone.

146. The device of claim 123 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A8.

147. The device of claim 123 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding an epitope existing on S100A9.

148. The device of claim 123 wherein the labeled polyclonal anti-calprotectin antibody is an antibody specifically binding a conformational epitope incorporating both S100A8 and S100A9.

149. The device of claim 123 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with all of calprotectin, isolated S100A8, and isolated S100A9.

150. The device of claim 123 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A8 but not at all or to a significantly lesser degree with isolated S100A9.

151. The device of claim 123 wherein the labeled polyclonal anti-calprotectin antibody is a labeled polyclonal antibody that reacts strongly with both calprotectin and isolated S100A9 but not at all or to a significantly lesser degree with isolated S100A8.

152. A method for determining the concentration of calprotectin in a sample comprising the steps of:

(a) applying the sample to the device of claim 123;

(b) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody; and

(c) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone.

153. The method of claim 152 wherein the method further comprises the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

154. A method of detecting or diagnosing the presence of active inflammatory bowel disease comprising the steps of:

(a) applying a sample that may contain calprotectin to the device of claim 123;

(b) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any

S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(c) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone; and

(d) determining the presence or absence of active inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case active inflammatory bowel disease is present in the patient, or is less than 50 mg/kg, in which case active inflammatory bowel disease is absent in the patient.

155. The method of claim 154 wherein the method further comprises the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

156. A method of determining whether a patient with inflammatory bowel disease who has been in remission is at risk of suffering a relapse comprising the steps of:

(a) applying a sample that may contain calprotectin to the device of claim 123;

(b) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(c) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone; and

(d) determining the risk of relapse of inflammatory bowel disease in the patient according to whether the calprotectin concentration in the sample is at least 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease exists, or is less than 50 mg/kg, in which case a risk of relapse of inflammatory bowel disease does not exist.

157. The method of claim 156 wherein the method further comprises the steps of constructing a standard curve and determining the concentration of calprotectin by comparison with the standard curve.

158. A method of performing an immunoassay for S100A9 polypeptide and for a related antigen to determine whether inflammatory bowel disease exists in a subject, the method comprising the steps of:

(a) providing a stool sample from a subject;

(b) applying the sample to the device of claim 123;

(c) allowing the sample and the labeled anti-calprotectin antibody to migrate through the solid support for lateral flow and to the detection zone at which any S100A9 polypeptide in the sample binds to the immobilized anti-S100A9 antibody in the detection zone and to the labeled anti-calprotectin antibody;

(d) performing an immunoassay for a related antigen;

(e) determining the quantity of labeled anti-calprotectin antibody bound to the detection zone to determine the concentration of calprotectin in the sample by quantitation of the antibody-antigen-antibody sandwich at the detection zone;

(f) determining the concentration of the related antigen in the stool sample from the results of the immunoassay for the related antigen; and

(g) comparing the concentration of S100A9 polypeptide and the concentration of the related antigen in the stool sample with the cutoff values for S100A9 polypeptide and the related antigen to determine whether or not inflammatory bowel disease exists in the subject.

159. The method of claim 158 wherein the related antigen is S100A12.

160. The method of claim 158 wherein the related antigen is lactoferrin.

161. The method of claim 158 wherein the immunoassay for the related antigen is performed in a competitive immunoassay.

162. The method of claim 158 wherein the immunoassay for the related antigen is performed in a sandwich immunoassay.

163. A kit for immunoassay of S100A9 and diagnosis, screening, or monitoring of inflammatory bowel disease comprising, separately packaged:

(a) a solid support for performing a lateral flow sandwich immunoassay for detection of S100A9 polypeptide; and

(b) a labeled anti-calprotectin antibody to be applied to the solid support.

164. The kit of claim 163 wherein the kit includes a single solid support and a quantity of labeled anti-calprotectin antibody sufficient for a single assay.

165. The kit of claim 163 wherein the kit includes a plurality of solid supports and a quantity of labeled anti-calprotectin antibody sufficient for a plurality of assays such that the quantity of labeled anti-calprotectin antibody is sufficient for use with each of the solid supports included in the kit.

166. The kit of claim 163 wherein the label of the labeled anti-calprotectin antibody is an enzyme label and wherein the kit further comprises, separately packaged, a substrate for the enzyme label.

167. The kit of claim 163 further comprising, separately packaged, a set of S100A9 polypeptide standards of differing concentrations so that a standard curve for S100A9 polypeptide can be established.

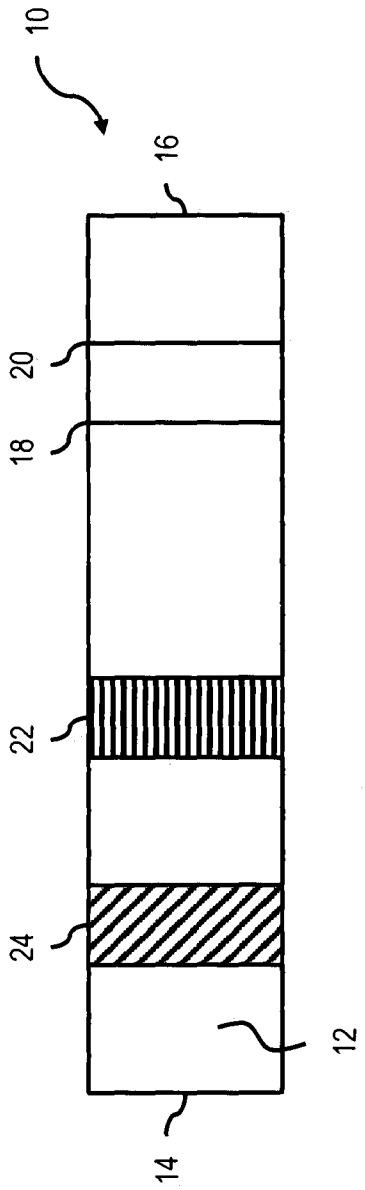


FIG. 1A

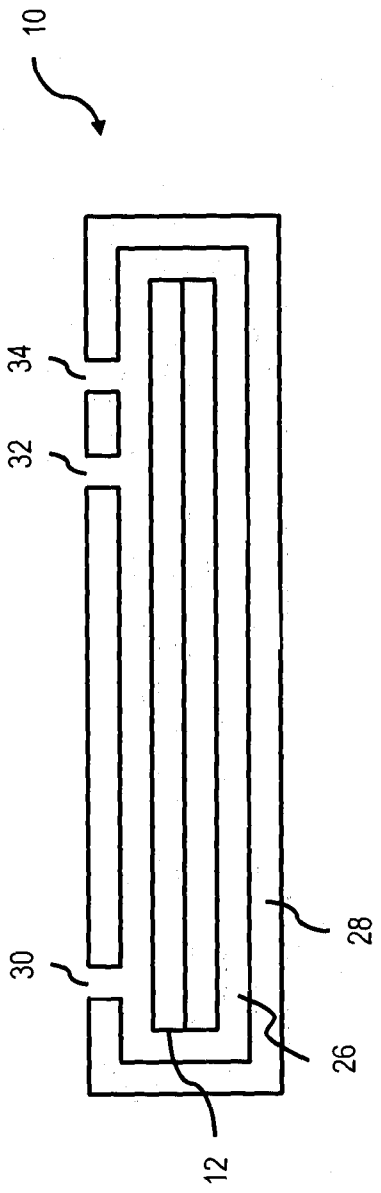


FIG. 1B

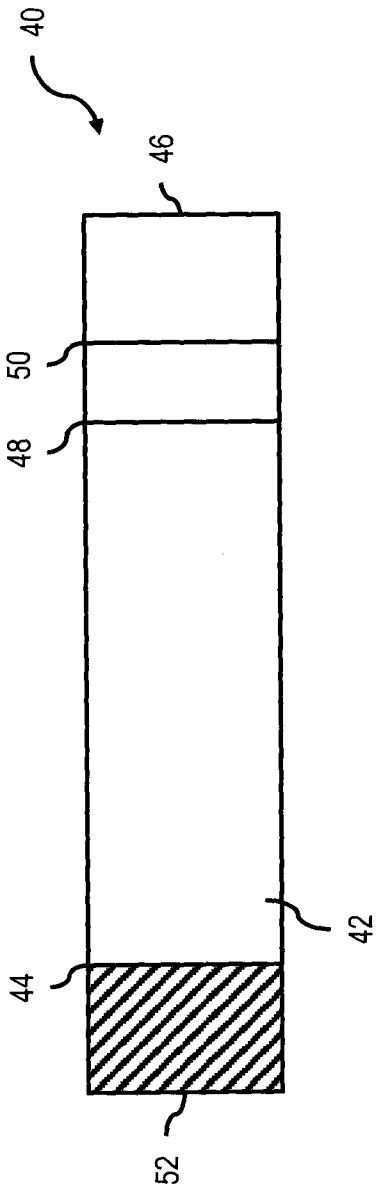


FIG. 2A

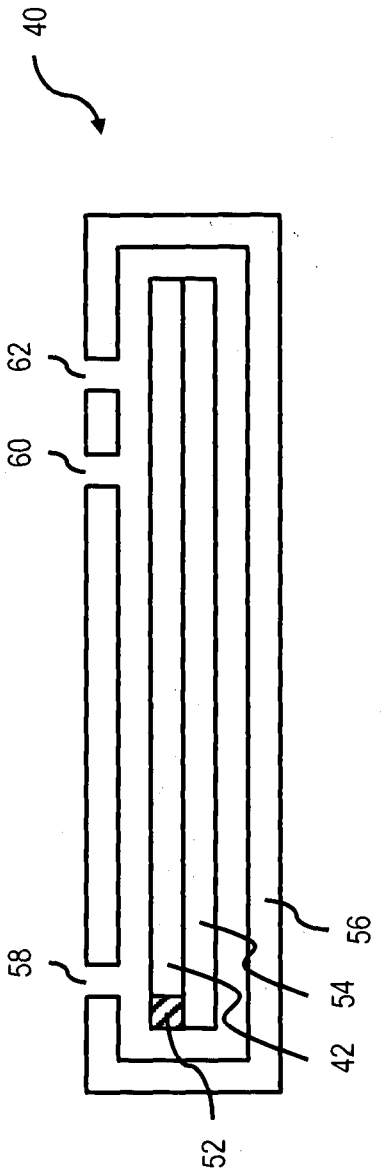


FIG. 2B

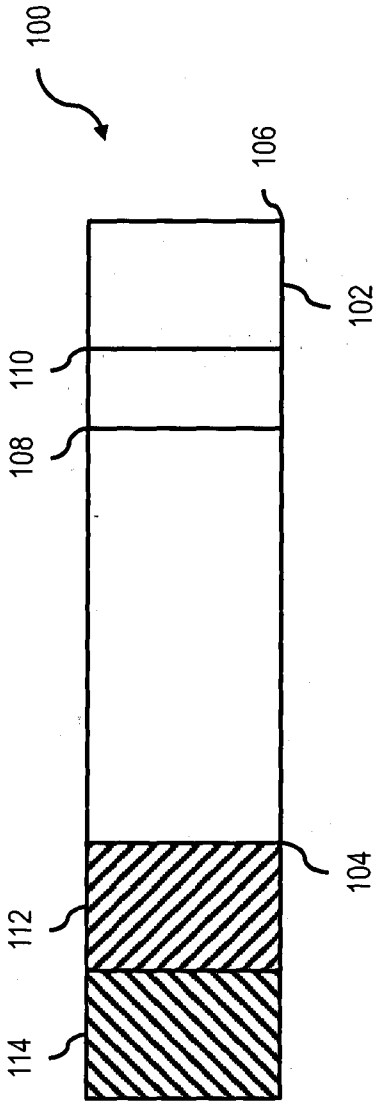


FIG. 3A

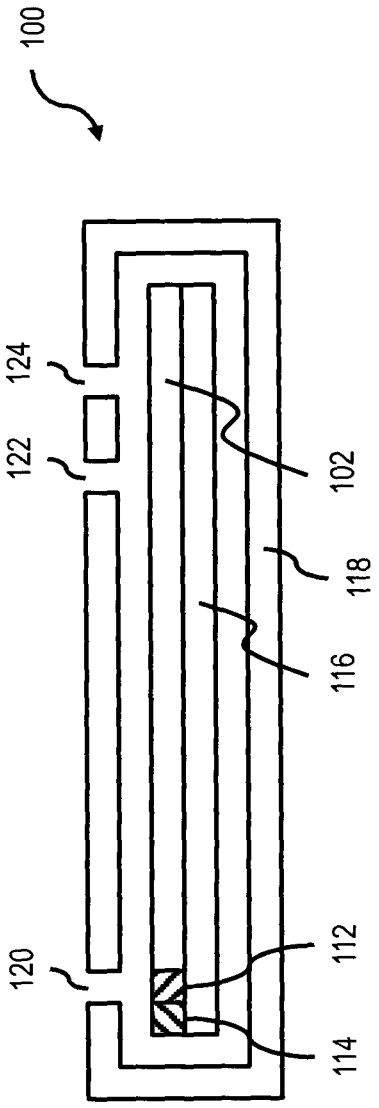


FIG. 3B

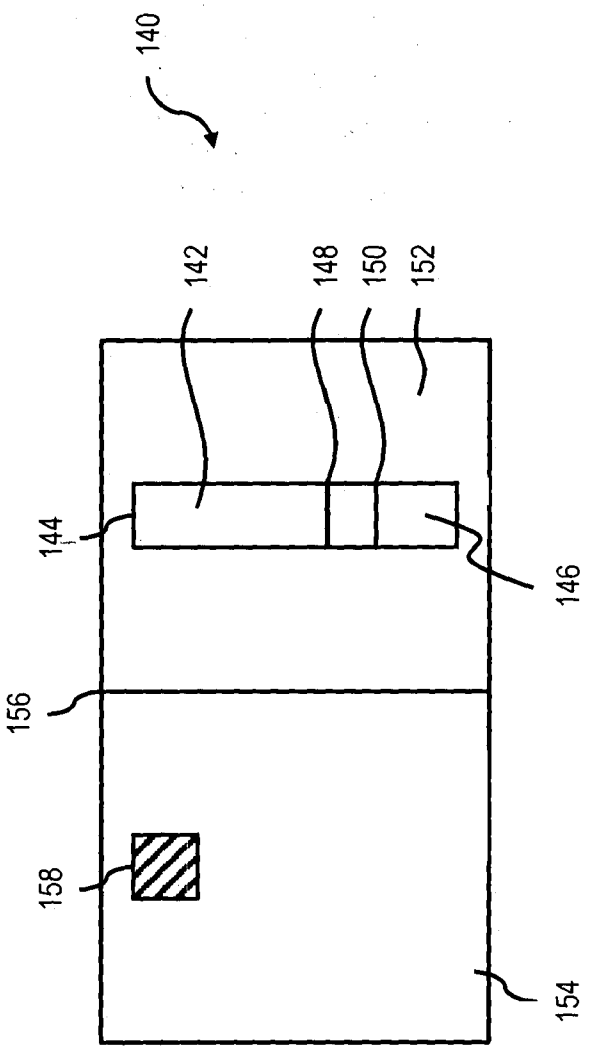


FIG. 4

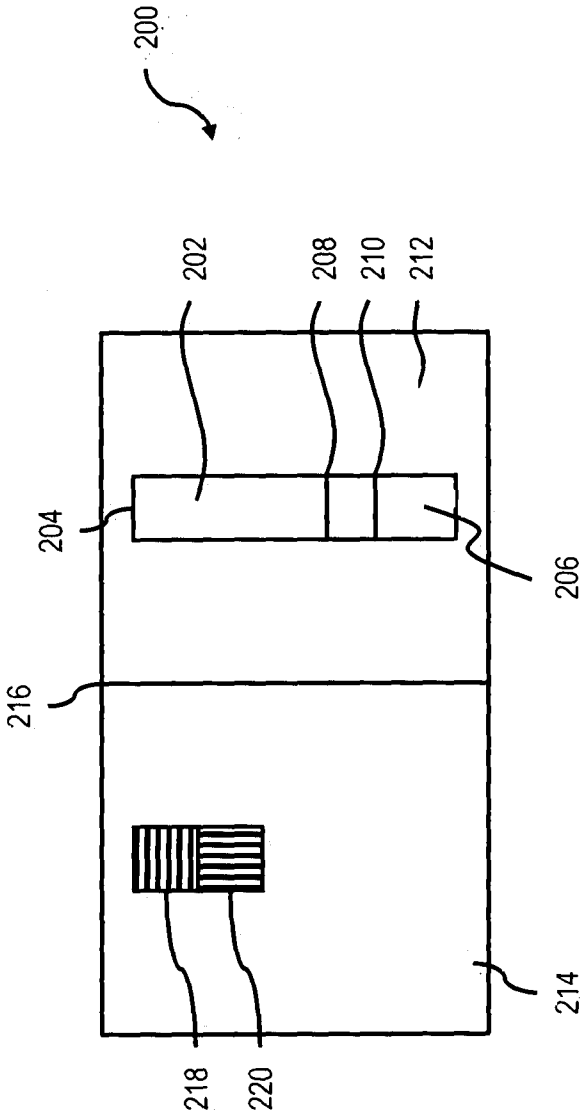


FIG. 5

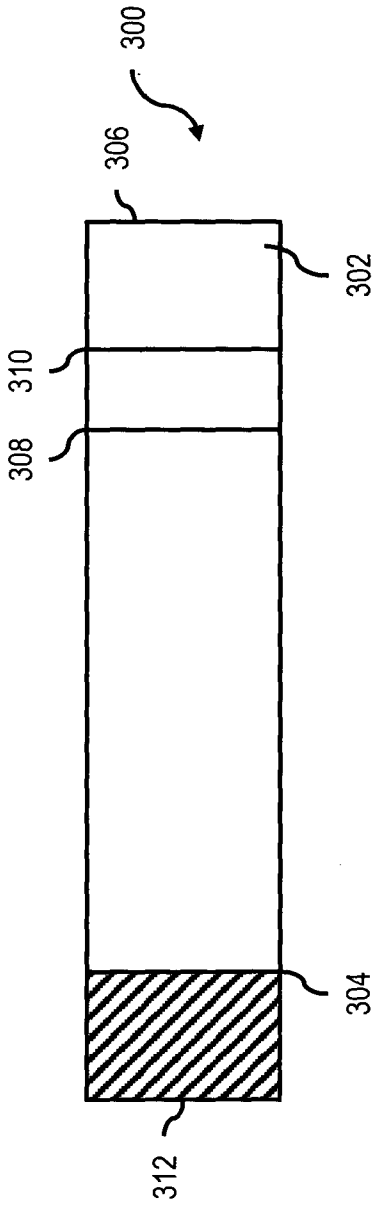


FIG. 6A

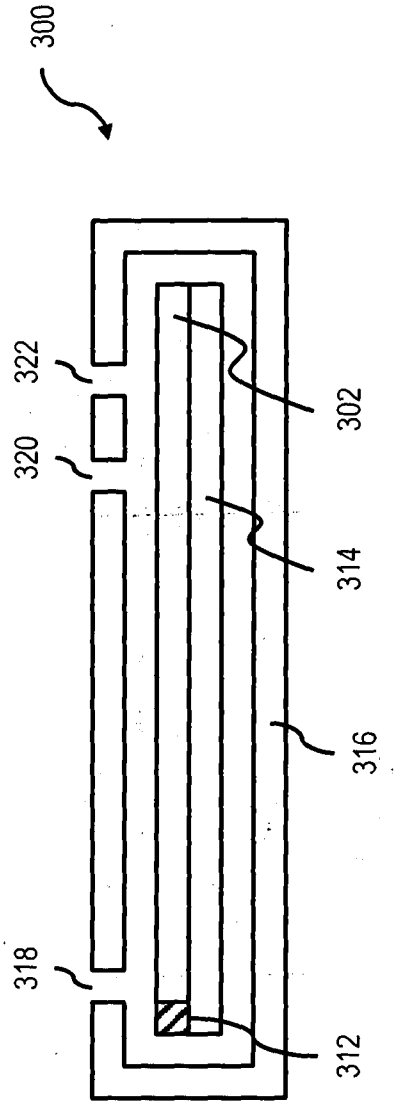


FIG. 6B

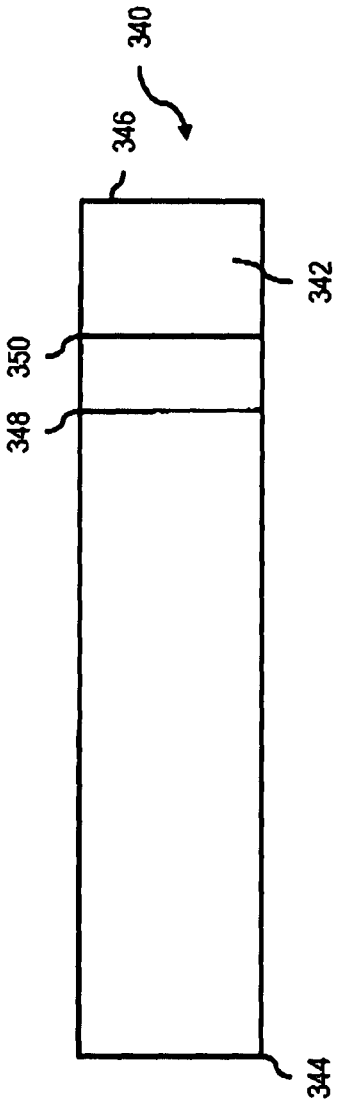


FIG. 7A

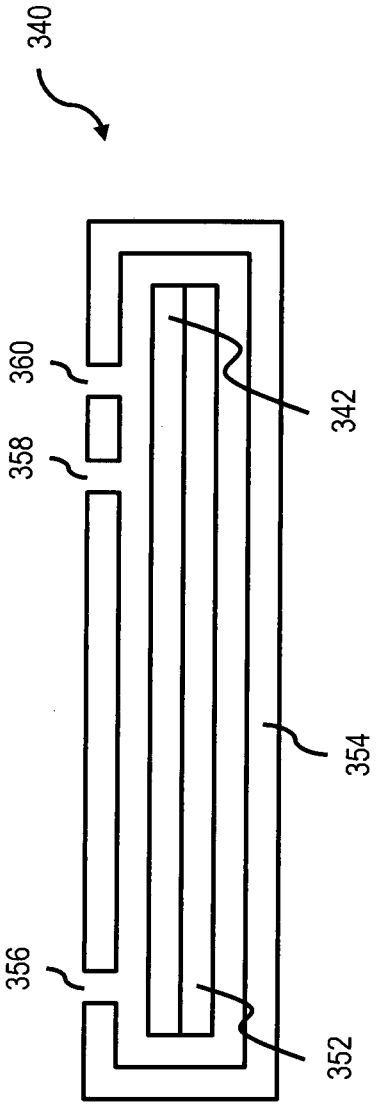


FIG. 7B

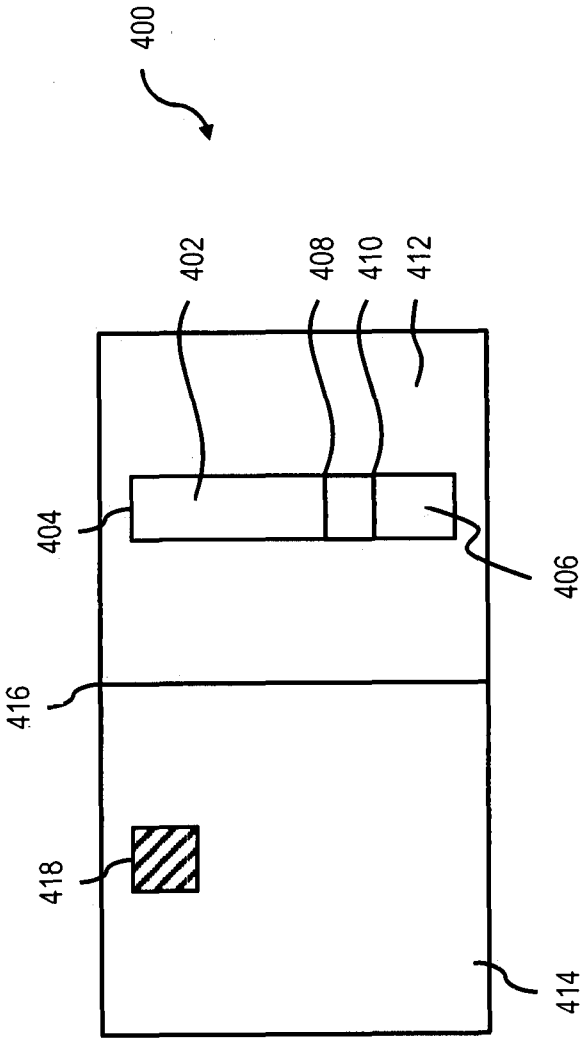
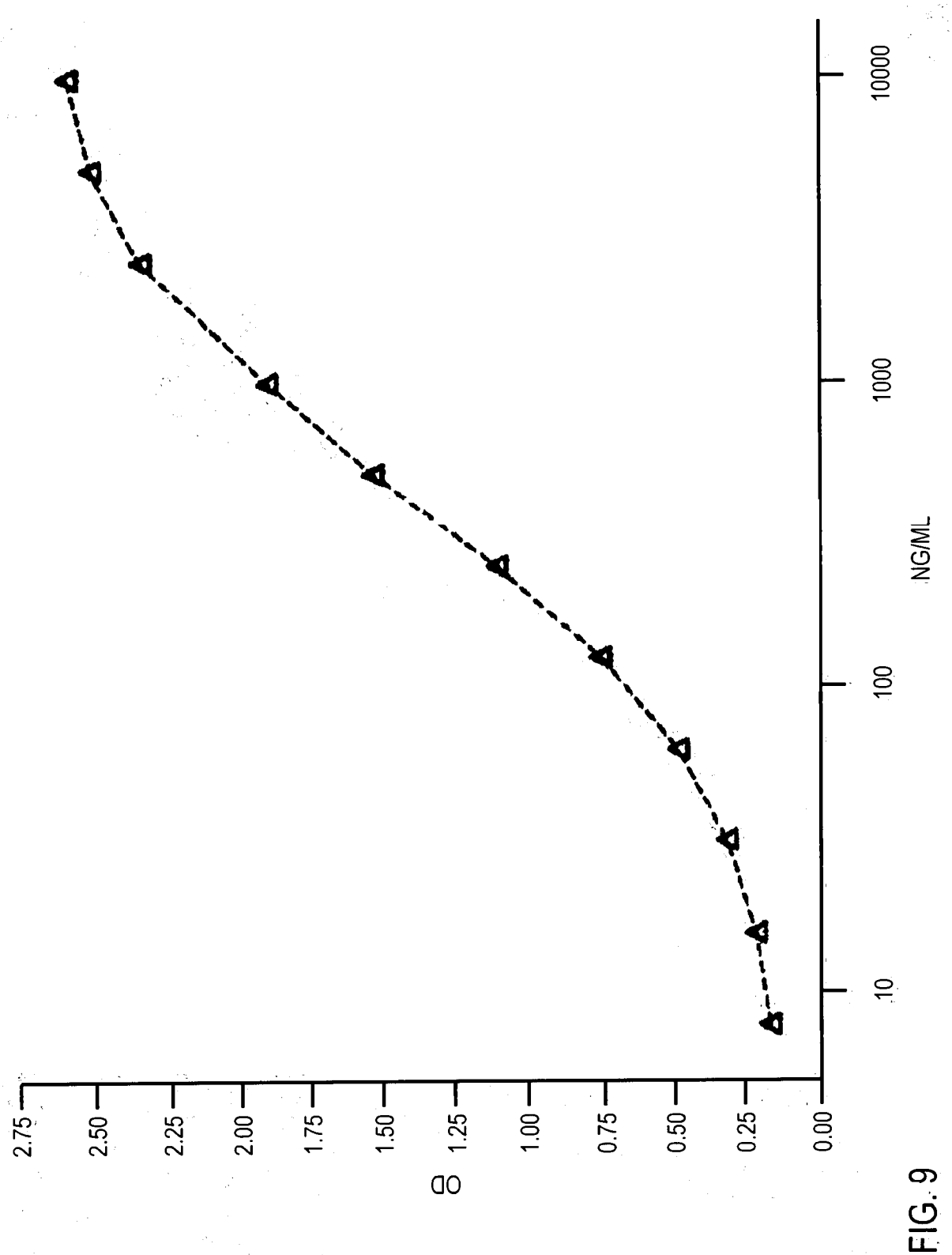


FIG. 8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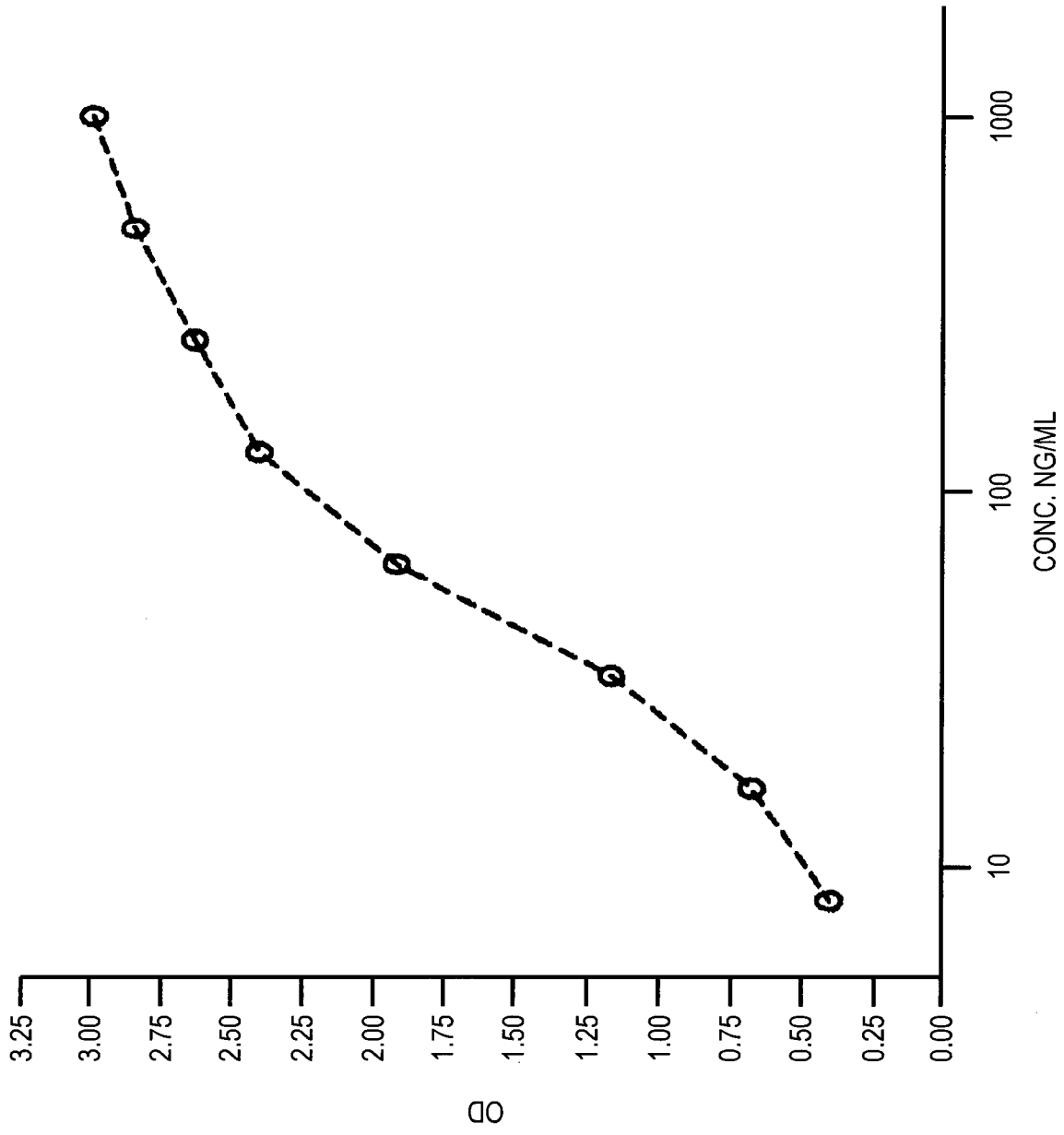
NG/ML

FIG. 9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CONC. NG/ML

FIG. 10

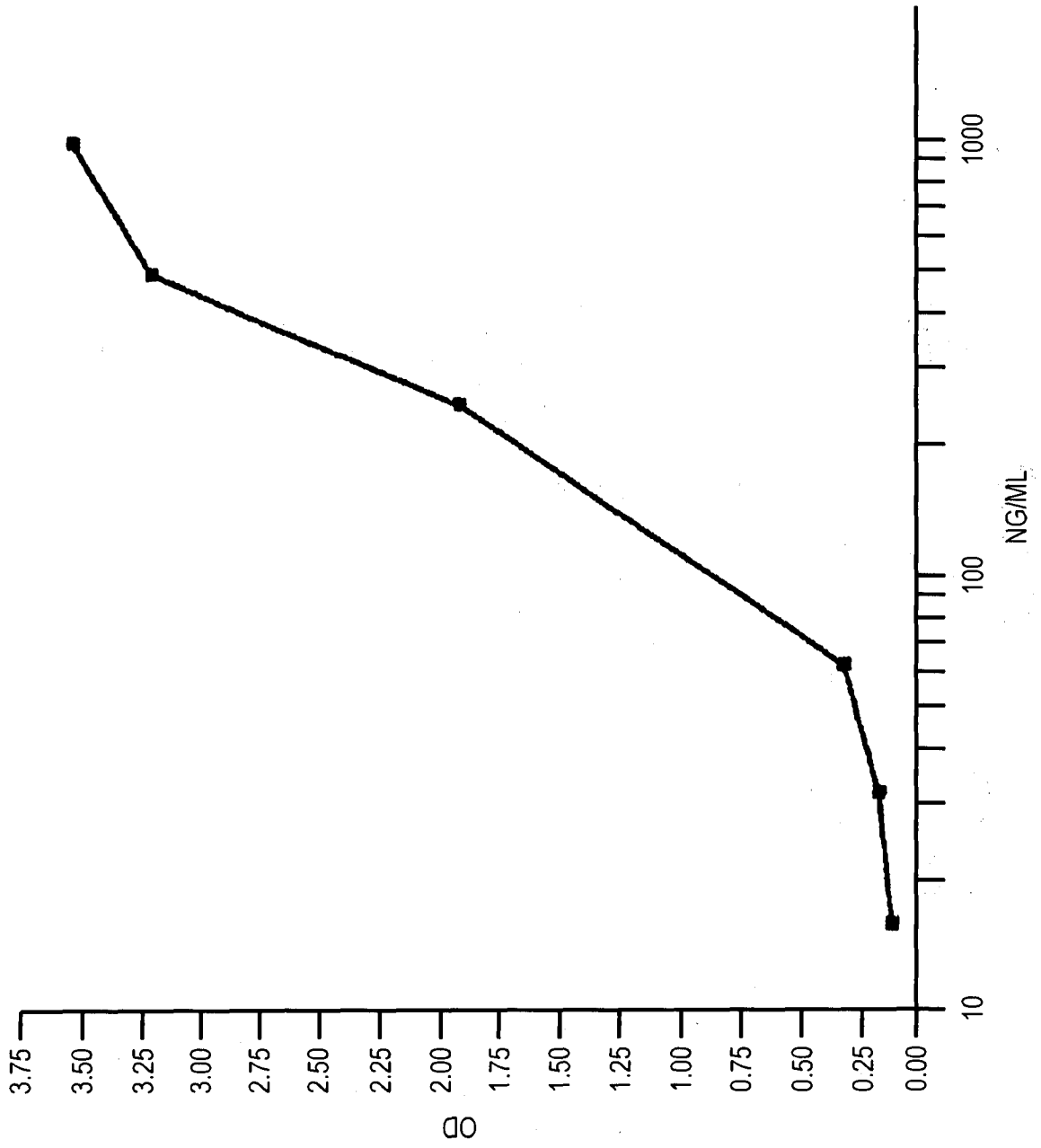
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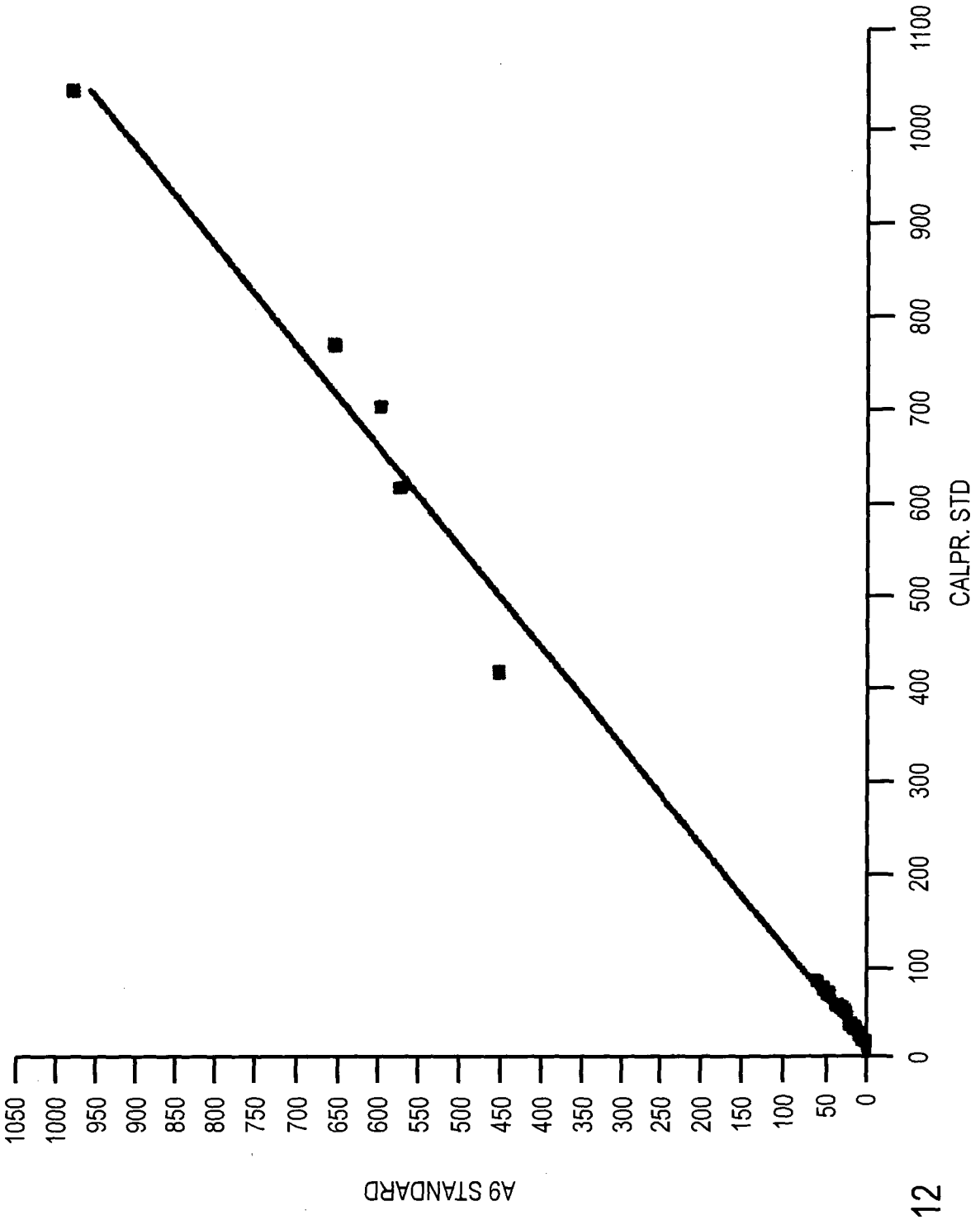
FIG. 11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CALPR. STD
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1100

FIG. 12