10966 HKS:PJW:PFB

P/00/008
Section 29(1)
Regulation 3.1(2)

AUSTRALIA
Patents Act 1990

NOTICE OF ENTITLEMENT

We, EPITOPE, INC.

of 15425 SW Koll Parkway, Beaverton, Oregon 97006, UNITED STATES OF AMERICA

being the Applicants in respect of the Application No. 50986/90, state the following:-

The persons nominated for the grant of the patent;

have, for the following reasons, gained entitlement from the actual inventors:

The Nominated Persons are entitled by Deed of Assignment between the Inventors and the Nominated Persons dated 14th April 1989.

The persons nominated for the grant of the patent are;

entitled to rely on the basic Applications listed on the patent request form.

The persons nominated for the grant of the patent are;

the Applicants of the Applications listed in the declaration under Article 8 of the PCT.

Dated this 29th day of July 1991

EPITOPE, INC.
By their Patent Attorneys
COLLISON & CO.

H K SCHULZE

AU9050986

(12) PATENT ABRIDGMENT (11) Document No. AU-B-50986/90
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 640635

(54) Title
AVIDIN-BIOTIN ASSISTED IMMUNOASSAY

International Patent Classification(s)
(51)[5] G01N 033/543 G01N 033/53 G01N 033/532

(21) Application No. : 50986/90 (22) Application Date : 25.01.90

(87) PCT Publication Number : WO90/08957

(30) Priority Data

(31) Number (32) Date (33) Country
302877 30.01.89 US UNITED STATES OF AMERICA

(43) Publication Date : 24.08.90

(44) Publication Date of Accepted Application : 02.09.93

(71) Applicant(s)
EPITOPE, INC.

(72) Inventor(s)
THOMAS THIEME; JACK H. FELLMAN; STEFAN GAVOJDEA; ADOLPH FERRO

(74) Attorney or Agent
COLLISON & CO , 117 King William Street, ADELAIDE SA 5000

(57) Claim

1. A method for detection of an antibody of interest in a body fluid containing said antibody of interest and other antibodies, comprising the steps of:

a. providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column;

b. contacting and incubating a test body fluid containing said antibody of interest and other antibodies with said protein-bound matrix of step (a) whereby said protein reacts with said antibodies to immobilize said antibodies, including said antibody of interest;

c. providing a solution of an antigen specific to said antibody of interest, said antigen being biotinylated;

d. contacting and incubating said biotinylated antigen of step (c) with the immobilized antibody of interest of step (b) whereby said biotinylated antigen reacts with said immobilized antibody of interest to immobilize said biotinylated antigen;

e. providing a solution of avidin, said avidin covalently linked to an enzyme;

f. contacting sand incubating said solution of step (e) with the immobilized biotinylated antigen-immobilized

.../2

antibody-protein bound matrix complex of step (d) whereby said enzyme-labelled avidin reacts with said matrix complex to immobilize said enzyme-labelled avidin;

g. contacting and incubating said immobilized complex of step (f) with a substrate solution wherein said enzyme linked to avidin induces a reaction with said substrate and produces a detectable reaction product on the immobilized complex; and

h. correlating said detectable reaction product on the immobilized complex in the column to the presence of said antibody of interest to be detected.

15. A method for detection of an antibody of interest in a body fluid containing said antibody and other antibodies, comprising the steps of:

a. providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column;

b. contacting and incubating a test body fluid containing said antibody of interest and other antibodies with a solution of an antigen specific for the antibody of interest, said antigen being biotinylated;

c. contacting and incubating said combined solution of step (b) with a solution of avidin, said avidin covalently linked to an enzyme;

d. contacting and incubating said combined solution of step (c) with a matrix of step (a) such that a complex of antibody-biotinylated antigen-enzyme labelled avidin is immobilized by said matrix;

e. contacting and incubating said immobilized complex of step (d) with a substrate solution wherein said enzyme linked to avidin induces a reaction with said substrate and produces a detectable reaction product on the immobilized complex; and

f. correlating said detectable reaction product on the immobilized complex in the column to the presence of said antibody to be detected.

OPI DATE 24/08/90 APPLN. ID 50986 / 90
PCT AOJP DATE 27/09/90 PCT NUMBER PCT/US90/00378

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : G01N 33/543, 33/53 A1

(11) International Publication Number: WO 90/08957

(43) International Publication Date: 9 August 1990 (09.08.90)

(21) International Application Number: PCT/US90/00378

(22) International Filing Date: 25 January 1990 (25.01.90)

(30) Priority data:
302,877 30 January 1989 (30.01.89) US

(71) Applicant: EPITOPE, INC. [US/US]; 15425 SW Koll Parkway, Beaverton, OR 97006 (US).

(72) Inventors: THIEME, Thomas ; 7020 Corvallis Road, Independence, OR 97351 (US). FERRO, Adolph ; 5868 Suncreek Drive, Lake Oswego, OR 97035 (US). FELLMAN, Jack, H. ; 7615 S.W. View Pt. Terrace, Portland, OR 97219 (US). GAVOJDEA, Stefan ; 18188-C N.W. Walker Road, Beaverton, OR 97006 (US).

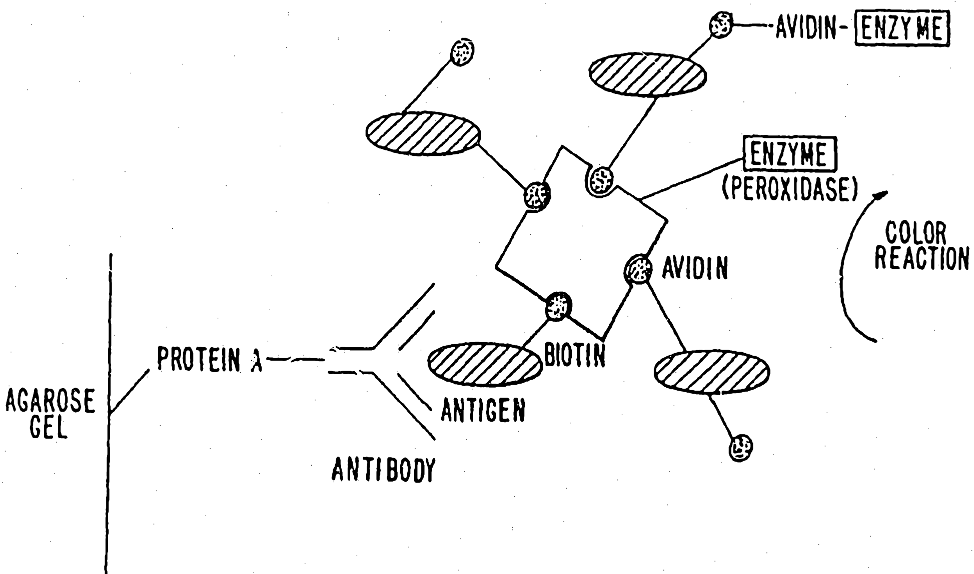
(74) Agents: PLAYER, William, E. et al.; Wegner & Bretschneider, 1233 20th Street N.W., P.O. Box 18218, Washington, DC 20036-8218 (US).

(81) Designated States: AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH (European patent), CM (OAPI patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GA (OAPI patent), GB (European patent), HU, IT (European patent), JP, KP, KR, LK, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL (European patent), NO, RO, SD, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

640635

(54) Title: AVIDIN-BIOTIN ASSISTED IMMUNOASSAY



(57) Abstract

A method for detection for screening and diagnostic purposes of antibodies in a body fluid by means of an antibody-binding protein covalently bound to a porous matrix trapped within a transparent column. A test fluid containing antibody is contacted with the protein-bound matrix whereby the protein reacts with to immobilize any antibody present in the test fluid. A biotinylated antigen of interest is contacted with the immobilized antibody whereby the biotinylated antigen binds to the immobilized antibody. Avidin covalently linked to an enzyme is contacted with the immobilized biotinylated antigen-immobilized antibody-protein bound matrix complex whereby the enzyme-labelled avidin binds to the immobilized complex. This immobilized complex is contacted with a substrate solution wherein the enzyme linked to avidin catalyzes a reaction of the substrate and produces a detectable reaction product which correlates to the presence of the antibody to be detected.

AVIDIN-BIOTIN ASSISTED IMMUNOASSAY

BACKGROUND OF THE INVENTION

This invention relates to the detection for screening and diagnostic purposes of antibodies in body fluids such as saliva, urine, tears, serum or plasma. In particular, this invention relates to the detection of antibodies which are present in low amounts in such fluids or where small amounts of such fluids are available and yet the antibodies present have antigenic specificities characteristics of particular disease states, and are of diagnostic value.

Body fluids of mammals, such as serum, plasma, saliva, tears, urine, milk, seminal fluid, synovial fluid, etc. can contain antibodies which are useful in the diagnosis of diseases, including those of bacterial and viral infection and of autoimmune origin. Body fluids, such as saliva, have significant advantage over serum and plasma as sources of these diagnostically valuable antibodies since they can be obtained without the facilities and hazards attendant with the taking of blood samples. However, often the concentration of antibodies is so low in these fluids as to make conventional tests for antibodies impractical.

Saliva, in particular, presents problems as a diagnostic indicator. These problems stem from the low concentration of antibodies in saliva and the inconvenience of collecting and processing quantities of saliva sufficient for a reliable diagnostic test, which can be quickly performed.

SUMMARY OF THE INVENTION

In one aspect, the present invention is a method for detection of antibody in a body fluid comprising the steps of providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column; contacting and incubating a test fluid containing said antibody with said protein-bound matrix whereby said protein reacts with said antibody to

immobilize said antibody; contacting and incubating a biotinylated antigen of interest with the immobilized antibody whereby said biotinylated antigen reacts with said immobilized antibody to immobilize said biotinylated 5 antigen; contacting and incubating said solution of avidin, said avidin covalently linked to an enzyme, with the immobilized biotinylated antigen-immobilized antibody-protein bound matrix complex whereby said enzyme-labelled avidin reacts with said matrix complex to become 10 immobilized; contacting and incubating said immobilized complex with a substrate solution wherein said enzyme linked to avidin catalyzes a reaction of said substrate and produces a detectable reaction product; and correlating said detectable reaction product to the presence of said 15 antibody to be detected.

In another aspect, the invention is a method for detection of antibody in a body fluid, comprising the steps of providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a 20 transparent column; contacting and incubating a test fluid containing said antibody with a solution of an antigen of interest, said antigen being biotinylated; contacting and incubating said combined solution with a solution of avidin, said avidin covalently linked to an enzyme; 25 contacting and incubating said combined solution with the matrix such that a complex of antibody-biotinylated antigen- enzyme labelled avidin is immobilized by said matrix; contacting and incubating said immobilized complex with a substrate solution wherein said enzyme linked to 30 avidin catalyzes a reaction of said substrate and produces a detectable reaction product; and correlating said detectable reaction product to the presence of said antibody to be detected.

A further aspect of the invention is a method for detecting an antigen in a test biological fluid, comprising the steps of providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column; providing a solution of test fluid containing said antigen and containing a small amount of antibody against said antigen in aqueous buffer; contacting and incubating said solution with said matrix such that a complex of antibody and antigen is immobilized by said matrix; providing a solution of monoclonal antibody modified so that it is a Fab fragment, lacking the Fc portion which binds to protein A but retaining its ability to bind antigen, said antibody being biotinylated; contacting and incubating said solution with the immobilized complex such that an antibody-antigen-Fab antibody complex is immobilized by said matrix; providing a solution of avidin, said avidin covalently linked to an enzyme; contacting and incubating said solution with the immobilized antibody-antigen-Fab antibody complex whereby said enzyme-labelled avidin reacts with said matrix complex to immobilize said enzyme-labelled avidin; contacting and incubating said immobilized complex with a substrate solution wherein said enzyme linked to avidin catalyzes a reaction of said substrate and produces a detectable reaction product; and correlating said detectable reaction product to the presence of said antibody to be detected. The invention also contemplates a test kit comprising a transparent open-ended column; a pair of porous frits disposed within said column to define a space therebetween; and a porous matrix having covalently bound thereto an antibody-binding protein; said matrix being trapped in said space between said pair of porous frits. The kit may also comprise appropriate reagents such as sample dilution buffer, biotinylated antigen solution, anti-antigen

antibody, biotinylated Fab monoclonal antibody, enzyme-linked avidin solution, enzyme substrate, and control solutions.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 schematically illustrates the binding mechanism utilized by the present invention; and

Figure 2 diagrammatically represents the apparatus used in the present invention.

DESCRIPTION OF THE PREFERRED EMBODIMENT

10 A preferred embodiment of the present invention comprises a method of rapidly isolating antibodies from a body fluid by passing the fluid through a porous matrix to which is chemically bonded the substance protein A, a protein which rapidly binds to antibody molecules. The antibodies are 15 thus rapidly removed from the fluid as it filters through the matrix and the antibodies are concentrated in the matrix for the subsequent colorimetric assay for antigenic specificity. It is preferred that the porous matrix is a predetermined amount of a beaded agarose gel with 20 covalently bonded protein A which is placed in a column made of transparent material. The column has a porous frit at the bottom to retain the agarose gel while allowing the body fluid to quickly filter through the gel. The amount of protein A-containing gel in the column is sufficient to 25 bind an optimal amount of the antibodies in the body fluid and provide an adequate size surface for the subsequent colorimetric assay.

Protein A is a protein isolated from the cell wall of the bacteria staphylococcus aureus (Cowan strain 1). Protein A 30 (also called Type 1 Fc receptor) is available commercially as a protein isolated from this bacteria and also in a recombinant form expressed in other bacteria. Protein A can be covalently linked to agarose by any of several commonly used chemical reactions. Some examples of these

techniques are the following: Amino groups or carboxyl groups are introduced into the agarose matrix by the cyanogen bromide method with diamino alkanes (introduction of amino group) or amino carboxylic alkanes (introduction
5 of carboxyl groups). This method is described by M. Wilchek et al., "The purification of biologically active compounds by affinity chromatography," Methods of Biochemical Analysis, 23:347-385, 1986. These derivatized agarose gels are then coupled to carboxyl groups or amino
10 groups on the protein by the use of the commonly used carbodiimide chemistries or the use of such commonly used leaving groups as N-hydroxysuccinimide which is introduced into the amino derivatized agarose with O-bromoacetyl-N-hydroxysuccinimide. Other known techniques can be used to
15 covalently bind protein A to agarose or other polysaccharide matrices.

Other proteins which rapidly and avidly bind antibodies are available, and can be used instead of protein A for the purposes described in this application. These proteins
20 include certain proteins isolated from group A streptococci (Type II Fc receptor), protein G (Type III Fc receptor) isolated from most human C and G streptococcus strains, Type IV Fc receptor isolated from some strain G streptococcus strains, and Types V, VI Fc receptors
25 isolated from streptococcus zooepidimicus. Each of these proteins has certain advantages over the others in its strength of binding to different subclasses of immunoglobulins and immunoglobulins from different mammalian species.

30 Other types of porous matrices are suitable for the purposes of this invention if they have low non-specific binding of proteins and functional groups such as hydroxyl groups or unique chemistries which allow the covalent binding of protein A or other proteins which bind

antibodies. An example of such matrix is the methacrylamide, glycidyl methacrylate beaded polymer available from Röhm Pharma under the name "Eupergit C".

After binding of antibodies to the porous matrix as 5 indicated above, excess amounts of the body fluid are removed from the matrix by washing with a suitable aqueous buffer. The matrix with bound antibody is then used as a substrate to assay for the antigenic specificity of the bound antibody.

10 Several methods are available for the assay of the antigenic specificity of isolated antibodies. These include the use of enzymes such as horseradish peroxidase or alkaline phosphatase covalently linked to the antigens of interest. This technique is illustrated in T. Kitagawa 15 et al., "Enzyme coupled immunoassay of insulin using a novel coupling reagent," Journal of Biochemistry, 79:233-236, 1976. These enzymes are used to develop color reactions which indicate the presence of the antigen. Another method which is available is the covalent binding 20 of biotin to the antigen as in G.R. Dreesman et al., U.S. Patent 4,535,057; D.A. Fuccillo, "Application of the avidin-biotin technique in microbiology," Bio-Techniques, 3:494-501, 1985; and M. Wilchek et al., "The use of the avidin-biotin complex as a tool in molecular biology," 25 Methods of Biochemical Analysis, 26:1-45. Biotin (also known as Vitamin H) has the following chemical structure:

30

It is bound with very high avidity (Kd 10-15M) by the protein avidin. Four biotin molecules are bound per avidin molecule. When enzymes such as horseradish peroxidase or alkaline phosphatase are chemically linked to the avidin

molecule, the avidin-biotin interaction can constitute a molecular bridge between the antigen of interest and the enzyme which is used to develop the color reaction that indicates the presence of the antigen. Because each avidin molecule has four biotin binding sites, the number of enzyme molecules per antibody molecule is increased in the presence of excess biotinylated antigen and the sensitivity of the assay is increased.

The chemical bond between the antigen and biotin molecule can be formed by a number of different chemical reaction sequences which are available. These reactions typically utilize N-hydroxysuccinimidyl or iodo leaving groups to derivative amino groups in the protein structures of the antigen. Typical reagents are shown below:

Na.OS ... N–O–C(=O)–$(CH_2)_2$–S–S–$(CH_2)_2$–N–C(=O)–$(CH_2)_4$– [biotin ring: S, NH, O, NH]

I–CH_2–C(=O)–N(H)–$(CH_2)_6$–N(H)–C(=O)–$(CH_2)_4$– [biotin ring: S, NH, O, NH]

[succinimide ring, O, O] N–O–C(=O)–$(CH_2)_5$–N–C(=O)–$(CH_2)_4$– [biotin ring: S, N, O, NH]

Carbohydrate antigens can be biotinylated by the reaction of biotin hydrazide with aldehyde groups produced in polysaccharide structures after reaction with periodate. The selection of the best biotinylation system among these 5 and other available chemical reactions depends on the particular nature of the antigen being tested.

The present invention represents a unique application of the use of the biotin-avidin reaction to link antigen with an enzyme detection system. The unique use lies in the 10 fact that the antigen has been immobilized as it passes through a porous matrix containing specific antibody which has in turn been immobilized by protein A as shown in Figure 1 which illustrates schematically the system.

In the presence of excess biotinylated antigen, many 15 avidin-enzyme conjugates can bind to the gel for every antibody immobilized by protein A. This gives the technique its high sensitivity.

The enzyme coupled to the avidin determines the colorimetric assay used in the detection of antigen, which 20 ultimately is a test for the presence of certain types of antibody in the biological fluid. Two of the more commonly used enzymes are alkaline phosphatase and horseradish peroxidase. These enzymes may be chemically bonded and linked to the avidin molecule utilizing commonly used 25 chemical pathways for protein; protein covalent linkage includes the use of glutaraldehyde or the use of other homobifunctional and heterobifunctional reagents such as disuccinimidyl suberate and succinimidyl-N-(4-carboxy-cyclohexylmethyl)-maleimide (S. Yoshitake et al., 30 "Conjugation of glucose oxidase from Aspergillus niger and rabbit antibodies using N-hydroxy-succinimide ester of N-(4-carboxy-cyclohexylmethyl)-maleimide," Eur. J. Biochem, 101:395-399). These chemistries utilize the reactivity of the amino group of proteins with the succinimidyl ester,

and the reactivity of the free SH group of proteins with the maleimide moiety.

If horseradish peroxidase is used as the detection enzyme, typical substrates would be diaminobenzidine, 4-chloro-1-naphthol, or 3,3',5,5'-tetramethylbenzidine(7). When any of these materials is mixed with hydrogen peroxide and exposed to the enzyme, a darkly colored polymeric material is formed. This colored product is fast, i.e., insoluble and precipitating on the solid support. The production of this colored precipitate indicates the presence of the enzyme and in the context of this invention the presence of antibodies specific for the antigen being investigated. Other materials producing highly colored precipitates are used to indicate the presence of alkaline phosphatase such as a mixture of 4-chloro-2-methylaniline and 3-hydroxy-2-naphthoic acid 4' chloro-2' methyl anilide phosphate, or a mixture of 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium.

In the present invention, the capture of antibodies by the Protein A attached to the insoluble matrix occurs in minutes with a total assay time of less than 15 minutes. The use of the column technique also lends speed and convenience to the present invention, and permits the concentration of antibodies from a large volume of fluid.

EXAMPLE I

The following is a typical and practical use of the ideas and techniques which are the basis of this invention: Crosslinked agarose consisting of beads (75-300 micron diameter) to which protein A has been linked by the techniques indicated above (either by the producer or a commercial supplier, e.g., Bio Rad Corp. Product 1536154) is washed in phosphate buffered saline (PBS) and incubated for 12 hours at 4°C as a 25% suspension in PBS containing 0.75% gelatin, 0.3% Tween 20 detergent. This reduces non-

specific binding of proteins to the agarose. The agarose-protein A beads are again washed in PBS. As shown in Figure 2, a clean polystyrene column 10 (8 mm I.D. x 102 mm) with a volume of 6 milliliters and a porous (70 µm pore size) polyethylene disk (frit) 12 at the bottom is used (e.g., Pierce Chemical Co. product 29920). A cap 14 is used on the bottom tip of the column to control the flow of liquid through the column. Columns of other dimensions which fulfill the basic requirements of the invention are usable. Two hundred microliters of the prepared agarose-protein A beads 16 are placed on the frit at the bottom of the column with adequate PBS for transfer. A second frit 18 is placed on top of the settled agarose beads resulting in a 4 mm thick disk of agarose beads sandwiched between 2 porous frits. The column described here fulfills the essential requirement of a device which will (1) contain an adequate quantity of agarose-protein A beads, (2) allow easy observation of the beads, (3) allow the flow of a body fluid to be tested through the beads with adequate contact surface between solutes in the body fluid and the protein A attached to the beads, (4) allow the sequential addition and washing out of the reagents used to perform the colorimetric tests of the antibodies in the body fluid.

After placing the agarose-protein A beads in the column as described, about one milliliter of the body fluid to be tested is diluted with 2 milliliters of an aqueous buffer which is 0.25M in TRIS (Tris (hydroxy methyl amino methane)), 0.37% gelatin, 0.30% Tween 20, 0.075M NaCl, and 0.01M in sodium phosphate. This buffer is called "diluent buffer" and has a pH of about 8.0. The diluted body fluid is placed in the column on the top of the upper frit and allowed to drain through the column by removing the bottom cap (3 to 5 minutes for this step). The column is then washed twice with 3 ml. portions of diluent buffer (3

minutes per wash). Ten micrograms of biotinylated antigen (prepared by producer) is added to 2 ml. of diluent buffer and drained through the column (2 minutes). Additional sensitivity can be obtained by preincubating antigen with body fluid for 5 minutes and then draining through the column. This is followed by 2 x 3 ml. washes with diluent buffer (3 minutes each). Twenty micrograms of avidin-peroxidase conjugate (prepared by producer or commercial supplier, e.g., Sigma Chemical Co. product 3151) is dissolved in 2 ml. diluent buffer and drained through the column (2 minutes). Again the column is washed 2 times with 3 ml. of diluent buffer (3 minutes each). These washes are followed by passing 2 ml. of a substrate solution through the column. The substrate solution can be 0.5 mg/ml. of 3,3 diaminobenzidine in PBS containing 0.00004 percent H_2O_2 (2 minutes). The extent of darkening of the gel at this point indicates the presence of antibodies in the biological fluid specific for the test antigen. An additional wash with water allows the test column to be capped and stored at refrigerator temperature with stable color development for several days.

Controls consist of columns run at the same time with known positive and negative samples of body fluids or suitable solutions of positive and negative antibodies. Alternative or additional controls for the purpose of color comparison can be agarose without protein A (negative) or agarose with biotin coupled to it. Biotin, which contains a carboxylic acid, can be coupled to agarose by the same chemical reactions used to couple protein A to agarose. These color-comparison-controls can be run in the same column as the test by simply placing the additional aliquots of agarose in the column with the agarose-protein A; each agarose aliquot is separated from the others by a polyethylene frit.

EXAMPLE II

The procedure described in Example I for assaying 1 ml. of body fluid takes about 27 minutes. This time can be significantly reduced by the following modification: One
5 milliliter of body fluid is combined with 2 ml. of diluent buffer. Ten micrograms of biotin-antigen complex are added followed by incubation for 2 minutes at room temperature. Twenty micrograms of avidin-peroxidase complex are added and the mixture is immediately passed through the column (3
10 minutes). This is followed by 2 washes with 3 ml. of diluent buffer (6 minutes) and color development with 2 ml. of diaminobenzidine-H_2O_2 substrate solution (2 minutes). The total assay time with this modified procedure is about 13 minutes and the sensitivity is equal to or greater than
15 that described earlier.

EXAMPLE III

Using the abbreviated column procedure of Example II, we have tested the saliva and serum of Acquired Immune Deficiency (AIDS) patients which are confirmed to have
20 serum antibodies against the Human Immunodeficiency Virus-1 (HIV-1) proteins (positive samples). Ten micrograms of biotinylated HIV viral lysate were used per column. Using 6 microliters of positive serum or 150 microliters of positive saliva, a near maximum dark brown color was
25 obtained on the agarose beads using the diaminobenzidine substrate. Control serum and saliva from uninfected persons gave essentially colorless agarose beads with the same procedure. The same results have been obtained using as antigen an HIV protein produced by expression of a
30 recombinant gene (commercial product, envelope peptide p121, Centocor, Inc.). Similar results have also been obtained using a viral lysate of the virus HTLV-1 and serum from infected patients.

In a like manner, this invention should be useful for the detection of disease specific antibody in body fluids of patients with infection by such agents as Hepatitis B and Herpes Simplex Type I and Type II.

5 Another format which fulfills the basic function of the column is to place the sandwich of frit-protein A-agarose-frit in a disposable plastic pipette tip. The frits wedge tightly against the walls of the tip, confining the agarose beads and allowing the flow of liquid through the beads. 10 When attached to the standard hand pipetting unit, the tip can be used to draw the body fluids and various reagents and washing fluids through the agarose beads.

EXAMPLE IV

In addition to its ability to detect antibodies specific 15 for HIV and other antigens, the present invention can be modified to directly detect the antigens of interest. Biotinylated antigen is omitted in this procedure. The biological fluid to be tested is added to blocking buffer in the column. Two micrograms of human antibody against 20 the antigen(s) is also added to this solution in the blocking buffer. After 5-10 minutes incubation, there is added 2 micrograms of a mouse monoclonal antibody that has been modified so that it is a Fab fragment (lacking the Fc part of the molecule which binds to Protein A, but 25 retaining its ability to bind antigen). (D.W. Dresser, "Assays for immunoglobulin-secreting cells," in D.M. Weir (Ed.), Handbook of Experimental Immunology, 34d ed., Blackwell Scientific Publications, Oxford; and J.J. Langone, "Protein A of Staphylococcus aureus and related 30 immunoglobulin receptors produced by Streptococci and Pneumonococci," Advances in Immunology, 32:157-252.) This modified monoclonal antibody is also biotinylated as previously described. The monoclonal antibody binds to those portions of the antigen which are not bound to the

human antibody. After another 5-10 minutes incubation, the avidin-enzyme detection reagent is added and the mixture drained through the column. After washing, chromogenic detection is carried out as before. The monoclonal antibody Fab fragment will only bind to the column, and thus color development will only be seen, if the antigen of interest is present. In contrast to other immunoassays for the direct detection of antigen, this technique captures antigen as an antigen-antibody complex by binding of antibody, including the antibody endogenously present in the biological sample. Also, larger volumes of biological fluid can be concentrated on a small detection surface.

Example V

This technique can also be adapted to biological fluids which lack endogenous antibodies against the antigen of interest and where there are no other sources of human antibody specific for the antigen. This would be the case, for example, in a plant tissue extract to be assayed for antigens of a plant pathogen. Here, two monoclonal antibodies specific for the antigens of the pathogen would be used. One antibody would be added to the extract to bind the antigen and mediate its binding to the immobilized Protein A. The second monoclonal would be made into a biotinylated Fab fragment and used as a detection antibody.

A specific potential application of this technique exists for the detection of _Corynebacterium sepedonicum_ infection of potatoes. _C. sepedonicum_ infection produces bacterial ring rot in potatoes, a disease of significant commercial impact. Stem and tuber extracts of affected plants contain many of these gram-positive bacteria. A number of monoclonal antibodies have been produced in mice to the antigens of _C. sepedonicum_ which can be used in an immunoassay for the organism. Extracts of suspect plant tissues are prepared according to standard techniques in

fluid containing detergent to maximize solubility of the bacterial antigens. Insoluble materials are removed by filtration and centrifugation. Between 1 and 5 mL of this extract are added to the column described above. The
5 column contains 1 mL of diluent buffer and 10 micrograms of a monoclonal antibody against a principal bacterial antigen. Ten micrograms of a second monoclonal antibody, biotinylated and present as a Fab fragment, are also present. This monoclonal antibody is specific for a second
10 epitope on the same bacterial antigen. After 5 minutes incubation, the mixture is drained through the column. The column is washed with diluent buffer as described above. Avidin linked to enzyme is then passed through the column followed by washes as previously described in Examples I
15 and II. Suitable chromogenic substrates for the enzyme are added and color development estimated in comparison to positive and negative controls to assess the presence of bacteria in the original tissues.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS

1. A method for detection of an antibody of interest in a body fluid containing said antibody of interest and other antibodies, comprising the steps of:

5 a. providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column;

b. contacting and incubating a test body fluid containing said antibody of interest and other antibodies with said
10 protein-bound matrix of step (a) whereby said protein reacts with said antibodies to immobilize said antibodies, including said antibody of interest;

c. providing a solution of an antigen specific to said antibody of interest, said antigen being biotinylated;

15 d. contacting and incubating said biotinylated antigen of step (c) with the immobilized antibody of interest of step (b) whereby said biotinylated antigen reacts with said immobilized antibody of interest to immobilize said biotinylated antigen;

20 e. providing a solution of avidin, said avidin covalently linked to an enzyme;

f. contacting sand incubating said solution of step (e) with the immobilized biotinylated antigen-immobilized antibody-protein bound matrix complex of step (d) whereby
25 said enzyme-labelled avidin reacts with said matrix complex to immobilize said enzyme-labelled avidin;

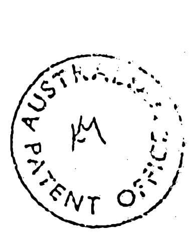
g. contacting and incubating said immobilized complex of step (f) with a substrate solution wherein said enzyme linked to avidin induces a reaction with said substrate and
30 produces a detectable reaction product on the immobilized complex; and

h. correlating said detectable reaction product on the immobilized complex in the column to the presence of said antibody of interest to be detected.

2. The method of claim 1, wherein said body fluid is saliva.

3. The method of claim 1, wherein said matrix is any porous material having low non-specific binding of proteins

5



and functional groups or other chemistries which allow binding or proteins that bind antibodies.

4. The method of claim 3, wherein said matrix is agarose.

5. The method of claim 4, wherein color-comparison controls are run in the same column using additional aliquots of agarose separated from one another with polyethylene disks.

6. The method of claim 1, wherein said protein is protein A.

7. The method of claim 1, wherein said protein is derived from sources chosen from the group of Types II, III, IV, V, and VI Fc receptor.

8. The method of claim 1, wherein after said test fluid is contacted and incubated with said protein-bound matrix, said matrix is washed.

9. The method of claim 1, wherein after said biotinylated antigen is contacted and incubated with said immobilized antibody, said matrix is washed.

10. The method of claim 1, wherein after said enzyme-linked avidin is contacted and incubated with said immobilized antigen complex, said matrix is washed.

11. The method of claim 1, wherein the column is open-ended.

12. The method of claim 1, wherein the column is polystyrene.

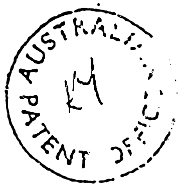
13. The method of claim 1, wherein the matrix is supported by a porous polyethylene disk.

14. The method of claim 1, wherein the test body fluid is diluted before application with an aqueous buffer.

15. A method for detection of an antibody of interest in a body fluid containing said antibody and other antibodies, comprising the steps of:

a. providing an antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column;

5



b. contacting and incubating a test body fluid containing said antibody of interest and other antibodies with a solution of an antigen specific for the antibody of interest, said antigen being biotinylated;

c. contacting and incubating said combined solution of step (b) with a solution of avidin, said avidin covalently linked to an enzyme;

d. contacting and incubating said combined solution of step (c) with a matrix of step (a) such that a complex of antibody-biotinylated antigen-enzyme labelled avidin is immobilized by said matrix;

e. contacting and incubating said immobilized complex of step (d) with a substrate solution wherein said enzyme linked to avidin induces a reaction with said substrate and produces a detectable reaction product on the immobilized complex; and

f. correlating said detectable reaction product on the immobilized complex in the column to the presence of said antibody to be detected.

16. The method of claim 15, wherein after said combined solution of step (c) is contacted and incubated with the matrix of step (a), said matrix is washed.

17. The method of claim 15, wherein the antigen is HIV-1.

18. The method of claim 1 or 15, wherein the assay is performed in a pipette tip.

19. A method for detecting an antigen in a test biological fluid containing said antigen, comprising the steps of:

a. providing an Fc receptor antibody-binding protein covalently bound to a porous matrix, said matrix being trapped within a transparent column;

b. providing a solution of test fluid containing said antigen and containing a small amount of antibody against said antigen in aqueous buffer;

c. contacting an incubating said solution of step 5 (b) with said matrix of step (a) such that a complex of antibody and antigen is immobilized by said matrix;

d. providing a solution of monoclonal antibody modified so that it is an Fab fragment, lacking the Fc portion which binds to the protein but retaining its ability to bind said antigen, said antibody being
5 biotinylated;

e. contacting and incubating said solution of step (d) with the immobilized complex of step (c) such that an antibody-antigen-Fab antibody complex is immobilized by said matrix;

10 f. providing a solution of avidin, said avidin covalently linked to an enzyme;

g. contacting and incubating said solution of step (f) with the immobilized antibody-antigen-Fab antibody complex of step (e) whereby said enzyme-labelled avidin
15 reacts with said matrix complex to immobilize said enzyme-labelled avidin;

h. contacting and incubating said immobilized complex of step (g) with a substrate solution wherein said enzyme linked to avidin induces a reaction with said
20 substrate and produces a detectable reaction product on the immobilized complex; and

i. correlating said detectable reaction product on the immobilized complex in the column to the presence of said antigen to be detected.

25 20. The method of claim 19, wherein after said enzyme-linked avidin is contacted and incubated with said immobilized complex of step (e), said matrix is washed.

21. The method of claim 19, wherein the anti-antigen antibody of step (b) and the anti-antigen antibody of step
30 (d) are specific for different epitopes of said antigen.

22. The method of claim 19, wherein the biological test fluid lacks endogenous antibodies against said antigen.

23. The method of claim 22, wherein the biological test fluid is a plant extract containing antigens of a plant pathogen.

24. The method of claim 23, wherein the antigen is a
5 bacterium.

25. The method of claim 24, wherein the bacterium is Corynebacterium sepedonicum.

~~26. A kit for detecting antibodies in saliva for~~ diagnostic tests comprising:

5 (a) a transparent open-ended column;

(b) a pair of porous frits disposed within said column to define a space therebetween;

(c) a porous matrix having covalently bound thereto an antibody-binding protein; and

10 (d) said matrix being trapped in said space between said pair of porous frits.

27. A kit as defined in claim 26, wherein said matrix comprises agarose beads.

28. A kit as defined in claim 26, further comprising a

15 removable tip at one end of said column.

29. A kit as defined in claim 26, further comprising the reagents: sample dilution buffer, biotinylated antigen, solution, enzyme-linked avidin solution, enzyme substrate, and control solutions.

20 30. A kit as defined in claim 26, further comprising the reagents: polyclonal or monoclonal anti-antigen antibody ~~and biotinylated Fab monoclonal antibody.~~

Dated this 10th day of January 1992

EPITOPE, INC.
25 By their Patent Attorneys
COLLISON & CO.

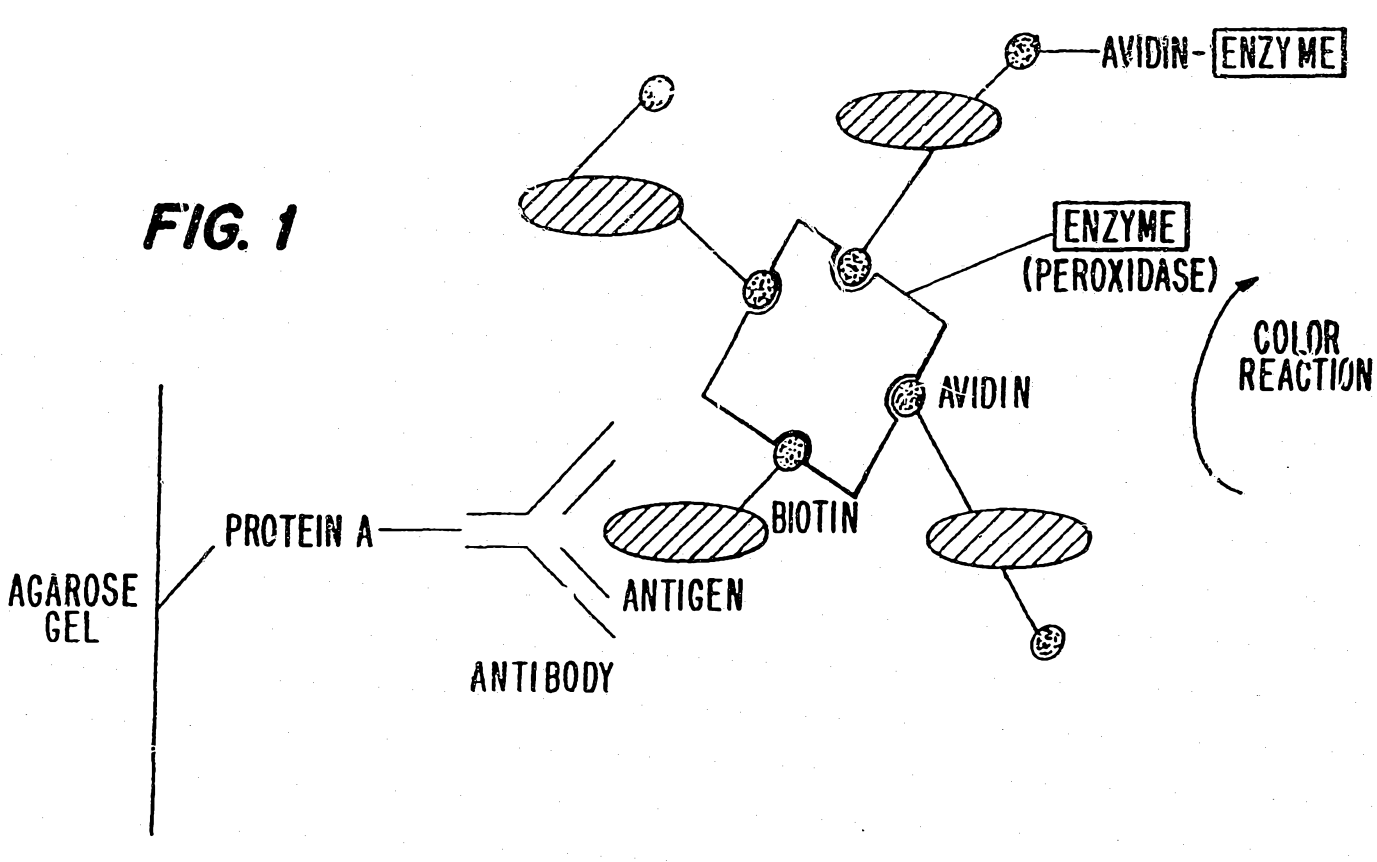
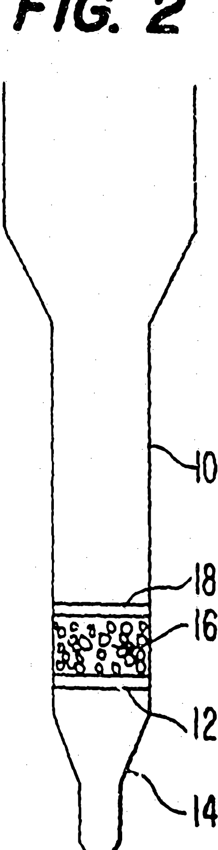


FIG. 1
AVIDIN-ENZYME
ENZYME
(PEROXIDASE)
COLOR
REACTION
AVIDIN
BIOTIN
PROTEIN A
AGAROSE
GEL
ANTIGEN
ANTIBODY

FIG. 2



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/00378

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: G 01 N 33/543, 33/53

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	EP, A1, 0021214 (AMES-YISSUM LIMITED, HAR HOTZVIM SANHEDRIA MURHEVET) 7 January 1981, see pages 18 and 3 and claims --	1-30
A	GB, A, 2109931 (AMANO PHARMACEUTICAL COMPANY LIMITED) 8 June 1983, see page 3, lines 43-80 --	1,15,19, 26
A	WO, A1, 8603589 (PHARMACIA AB) 19 June 1986, see pages 9-11, fig. 2 and claims --	1,3-6
A	EP, A1, 0060700 (BAUMAN DAVID S. ET AL.) 22 September 1982, see pages 18-19, figure 1 and examples	19,24
X		26,27
Y	--	28-30

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th May 1990	30.05.90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. Peis M. PEIS

Form PCT/ISA/210 (second sheet) (January 1985)

International Application No. PCT/US 90/00378

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	US, A, 4228237 (R C HEVEY ET AL.) 14 October 1980, see the whole document --	1-14
Y	EP, A1, 0071976 (F. HOFFMANN-LA ROCHE & CO. AG.) 16 February 1983, see the whole document --	15-18, 26
A	US, A, 4535057 (G R DREESMAN ET AL.) 13 August 1985, see the whole document --	1,15, 19
A	Dialog Information Services, file 154, Medline, Dialog Accession no. 05317155, Plebani A et al: "An avidin-biotin ELISA for the measurement of serum and secretory IgO", & J Immunol Methods Jul 6 1984, 71 (2) p 133-40. --	1,2
A	Dialog Information Services, File 154, Medline, Dialog accession no.05948028, Takatori T et al: "An ELISA using avidin-biotin complex for the determ ination of ABH group from saliva", & Forensic Sci Int May 1986, 31 (1) p 61-8. --	1,2
A	Patent Abstracts of Japan, Vol 4, No 144, C 27, abstract of JP 55- 92693, publ 1980-07-14 HEKISUTO JAPAN K.K. -- --------	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/US 90/00378

SA 34551

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/03/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0021214	07/01/81	AT-T- 2807	15/04/83
		AU-B- 516431	04/06/81
		AU-D- 5881780	12/03/81
		CA-A- 1140465	01/02/83
		JP-A- 56006163	22/01/81
		US-A- 4301139	17/11/81
GB-A- 2109931	08/06/83	DE-A- 3235516	21/04/83
		FR-A-B- 2519764	18/07/83
		JP-A- 58056696	04/04/83
WO-A1- 8603589	19/06/86	EP-A- 0204807	17/12/86
		JP-T- 62501447	11/06/87
		SE-B- 458643	17/04/89
		SE-A- 8406219	08/06/86
EP-A1- 0060700	22/09/82	AU-B- 560649	16/04/87
		AU-D- 8135482	16/09/82
		CA-A- 1184114	19/03/85
		JP-A- 57161647	05/10/82
		US-A- 4425438	10/01/84
US-A- 4228237	14/10/80	JP-A- 55092693	14/07/80
EP-A1- 0071976	16/02/83	AU-D- 8594382	10/02/83
		JP-A- 58030667	23/02/83
US-A- 4535057	13/08/85	AU-B- 567681	03/12/87
		AU-D- 1725883	05/07/84
		CA-C- 1224141	14/07/87
		EP-A- 0100955	22/02/84
		JP-A- 59042397	08/03/84

For more details about this annex : see Official Journal of the European patent Office, No. 12/82