(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
11 June 2009 (11.06.2009)

PCT

(10) International Publication Number
WO 2009/072708 A1

(51) International Patent Classification:
G01N 33/569 (2006.01) *G01N 33/53* (2006.01)
G01N 33/68 (2006.01) *G01N 33/531* (2006.01)

(21) International Application Number:
PCT/KR2008/003092

(22) International Filing Date: 2 June 2008 (02.06.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
10-2007-0125055
4 December 2007 (04.12.2007) KR

(71) Applicant *(for all designated States except US)*: **BIOLAND LTD.** [KR/KR]; 39-4, Songjeong-ri, Byeongcheon-myeon, Cheonan-si, Chungnam 330-863 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CHONG, Chom Kyu** [KR/KR]; 106-304, Wurim Feelyou Apt., Gak-ri, Ochang-eup, Cheongwon-gun, Chungbuk 363-883 (KR). **CHO, Nam Jeong** [KR/KR]; 26-39, Danggok-ri, Gangnae-myeon, Cheongwon-gun, Chungbuk 363-891 (KR). **PARK, Jong Jin** [KR/KR]; 101-303, Halla Dongbaek 2-cha Apt., Sinbang-dong, Cheonan-si, Chungnam 330-768 (KR). **CHEONG, Kwang Myun** [KR/KR]; Room 202, 36-2, Jeongja-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 440-300 (KR). **YOO, Jai Cheol** [KR/KR]; 602-905, Wurim Feelyou Apt., Gak-ri, Ochang-eup, Cheongwon-gun, Chungbuk 363-783 (KR). **LEE, Sang Oh** [KR/KR]; 104-2403, Halla Dongbaek 2-cha Apt., Sinbang-dong, Cheonan-si, Chungnam 330-768 (KR). **OH, Ha Na** [KR/KR]; 516-904, Bunpyeong 5-cha Apt., Bunpyeong-dong, Heungdeok-gu, Cheongju-si, Chungnam 361-770 (KR). **PARK, Sun Ock** [KR/KR]; 110-404, Wurim Feelyou 1-cha Apt., Gak-ri, Ochang-eup, Cheongwon-gun, Chungbuk 363-782 (KR).

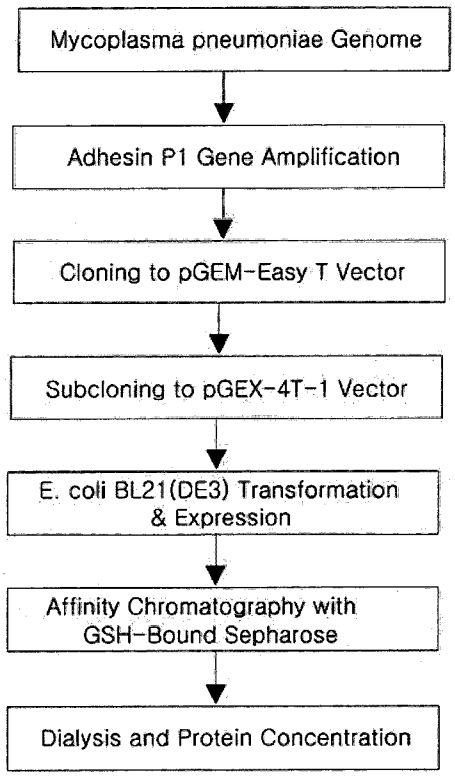
(74) Agent: **SON, Min**; 19th Floor, City Air Tower 159-9, Samseong-dong, Gangnam-gu, Seoul, 135-973 (KR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM,

[Continued on next page]

(54) Title: RECOMBINANT PROTEINS FOR THE DIAGNOSIS OF DISEASES INFECTED WITH MYCOPLASMA PNEUMONIAE AND DIAGNOSTIC KITS COMPRISING THE SAME

[Fig. 2]



(57) Abstract: Disclosed are a recombinant protein specific for antibodies against Mycoplasma pneumoniae, and a composition for the diagnosis of Mycoplasma pneumoniae infection, comprising the same. Also, a kit is provided for the diagnosis of Mycoplasma pneumoniae infection with the composition. A method for the diagnosis of Mycoplasma pneumonia using the kit is also disclosed.

WO 2009/072708 A1

AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

Description

RECOMBINANT PROTEINS FOR THE DIAGNOSIS OF DISEASES INFECTED WITH MYCOPLASMA PNEUMONIAE AND DIAGNOSTIC KITS COMPRISING THE SAME

Technical Field

[1] The present invention relates to recombinant proteins that specifically react with antibodies against *Mycoplasma pneumoniae* and methods for the diagnosis of *Mycoplasma pneumoniae* infection and *Mycoplasma pneumonia* using the same. Also, the present invention is concerned with hybridoma cells capable of producing antibodies against the recombinant proteins.

Background Art

[2] Pneumonia can result from a variety of causes, mainly infection with bacteria and viruses. Viral pneumonia is commonly caused by RSV (respiratory syncytial virus), adenovirus, and parainfluenza virus. Influenza viruses are the most common cause of pneumonia in children. As for bacterial pneumonia, it is also caused by various bacteria, including *Mycoplasma* spp.

[3] There are 16 recognized species of the genus *Mycoplasma* isolated from the human body. Of them, *Mycoplasma pneumoniae* is the predominant cause of respiratory tract infection, while *Ureaplasma urealyticum* and *Mycoplasma hominis* cause genitourinary tract infection and prenatal infection, respectively.

[4] Of patients who have mycoplasma infection, about 20% show no symptoms, 75% are afflicted with slight respiratory diseases, such as bronchitis, pharyngitis, etc., and 3 ~ 10% suffer from severe diseases such as pneumonia. In children, *Mycoplasma pneumoniae* is generally responsible for 10 ~ 40% of pneumonia cases. More cases of pneumonia occur during the late summer or the early fall months, rather than at other times of the year. Incidence differs from one year to another, with an epidemic cycle of four years.

[5] Lacking a cell wall, *Mycoplasma* is unaffected by some antibiotics such as penicillin or other beta-lactam antibiotics that target cell wall synthesis. For this reason, *Mycoplasma* infection must be treated with antibiotics that inhibit the synthesis of proteins or DNA, such as microlide, quinolone, and tetracycline. Although *Mycoplasma pneumoniae* is one of the most frequent causes of respiratory tract infection in children, the pneumonia caused thereby is one of the most seldom diagnosed diseases because of the difficult diagnosis thereof. As a matter of fact, the pneumonia caused by *Mycoplasma pneumoniae* is frequently misdiagnosed, resulting in the administration of inappropriate antibiotics.

[6]

[7] Standard diagnostic methodologies for diseases of *Mycoplasma pneumoniae* include the identification of bacteria through culture, serological assay, and genetic analysis by PCR. The identification of bacteria is difficult to apply in practice because it takes 7 ~ 21 days to identify bacteria through culture, and they are cultured at a rate of only 40 ~ 90%. Serological diagnosis of *Mycoplasma pneumoniae* infection is most widely used. However, a CF test (complement fixation test) for serological diagnosis requires a long period of time and is not sensitive enough. Recently, a gelatin microparticle agglutination test (e.g., SeroDia MycoII, Fujirebio) and ELISA (e.g., Platelia Myco IgM/IgG, Bio-Rad) have been generally applied to the determination of *Mycoplasma pneumoniae* infection. These two immunoassay methods, however, are difficult to conduct, require 3 ~ 4 hours for the completion thereof, and employ very expensive apparatus. Moreover, these methods do not allow point-of-care testing (POCT). Further, the concordance rate between the test results of the two methods is low, and thus the user is frequently confused as to the basis on which the determination is to be made. Therefore, active attempts have been made to develop POCT kits that effectively allow the serological detection of antibodies against *Mycoplasma pneumoniae*.

[8]

[9] A prerequisite to an effective assay for antibodies to *Mycoplasma pneumoniae* is to secure a protein which is of high antigenicity and evolutionarily well conserved among homologous species. Attachment sites of *Mycoplasma pneumoniae* include epidermal cells of the respiratory tract. For the attachment, various proteins, including adhesin P1 (170 kDa), adhesin P30 (30 kDa), adhesin P40 (40 kDa) and adhesin P90 (90 kDa), are collectively responsible. Of them, adhesin P1 is known to evoke potential antigenicity in humans and animals. However, adhesin P1 is difficult to recombinantly produce in E. coli because it has a large molecular weight and its gene contains 21 copies of a UGA sequence, which is recognized as a stop codon in E. coli. In fact, commercial diagnostic kits in the current market take advantage of an exudate of cultured *Mycoplasma pneumoniae*, suffering from the problem of being low in specificity.

[10]

[11] Leading to the present invention, intensive and thorough research into the diagnosis of mycoplasma pneumonia, conducted by the present invention, resulted in the finding that a recombinant, highly antigenic, and small protein can be expressed from a fragment of the gene encoding adhesion P1 of *Mycoplasma pneumoniae* and allow the preparation of a fused cell producing a monoclonal antibody thereto. Based on the antibody, a kit showing selectivity and specificity for *Mycoplasma pneumoniae* can be provided for the diagnosis of Mycoplasma pneumonia.

[12]

Disclosure of Invention

Technical Problem

[13] It is an object of the present invention to provide a recombinant protein of the adhesion P1 of *Mycoplasma pneumoniae*.

[14] It is another object of the present invention to provide a fused cell line capable of producing monoclonal antibodies to the recombinant adhesion P1 protein.

[15] It is a further object of the present invention to provide a composition for the diagnosis of *Mycoplasma pneumoniae* infection, comprising the antibody produced by the fused cell line and a kit for the diagnosis of *Mycoplasma pneumoniae* infection, comprising the composition.

[16]

Technical Solution

[17] In accordance with an aspect thereof, the present invention provides a recombinant adhesin P1 protein having high antigenicity to *Mycoplasma pneumoniae*.

[18] Adhesin P1 shows high antigenicity in humans and animals, but has a very large molecular weight and as many as 21 copies of a UGA sequence, which is recognized as a stop codon in E. coli. Selected in the present invention is a region of adhesion P1, which shows the following features: 1) no truncation upon protein expression because of the absence of UGA codons, 2) many epitopes with high antigenicity, as predicted by epitope prediction software, and 3) a net pI less than 7 and an average hydrophobicity index less than zero, allowing the expression thereof in a water-soluble form. In more detail, a region of adhesin P1 which 1) was predicted to have 20 or more MHC peptide binding motifs by the HLA peptide binding prediction program developed by Harvard University, 2) was found to have a molecular weight of 38.24 kDa and a pI of 4.72, as measured by the ExPASy system developed by the Swiss Institute of Bioinformatics, Switzerland, and 3) was found to have no transmembrane domains and an average hydrophobicity of -0.535, as measured by the SOSUI system (Nagoya University, Japan), was selected. The recombinant protein has the amino acid sequence of SEQ ID NO.1, encoded by a base sequence of SEQ ID NO. 2.

[19] In this regard, a gene was amplified using a pair of primers of SEQ ID NOS. 3 and 4 and was then cloned to a pGEM-T Easy vector. For the expression of the gene, a pGEX expression system (GE Healthcare, Uppsala, Sweden) was used. That is, a gene of adhesin P1 was sub-cloned to a pGEX-4T-1 expression vector, which was then transformed into E. coli BL21 (DE3). The recombinant adhesin P1 protein was over-expressed by inducing the resulting transformed E. coli with 1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside), followed by purification through affinity chro-

matography using GSH-Sepharose gel to afford a 64.0 kDa, pure recombinant adhesin P1 protein (FIG. 3).

[20]

[21] In accordance with another aspect thereof, the present invention provides a hybridoma cell line producing a monoclonal antibody to the recombinant adhesion P1 protein.

[22] In a preferable embodiment of the present invention, the spleen cells from a mouse immunized with the recombinant adhesin P1 protein were fused with myeloma cells to make a hybridoma cell line. To accomplish this, the pure recombinant adhesin P1 protein was peritoneally injected into a 6-8 week-old mouse from which the spleen was then excised. Spleen cells isolated from the spleen were fused with myeloma cells to make hybridoma cells. This mixture of cells was then diluted, and clones were grown from single parent cells. After tests for ability to bind to the antigen, the most productive and stable clone was then grown in culture medium to a high volume. The hybridoma cell line thus obtained was deposited at an international depositary authority, KCTC (Korean Collection for Type Cultures; Genetic Resources Center, Korean Research Institute of Bioscience and Biotechnology (KRIBB), Yusong-ku, Taejon, Korea) on November 15, 2007, and assigned accession number KCTC 11242 BP.

[23]

[24] In accordance with a further aspect thereof, the present invention provides novel monoclonal antibodies specific for the mycoplasma adhesin P1 for use in the diagnostic kit of the present invention.

[25] As used herein, the term "monoclonal antibodies" is intended to refer to highly specific antibodies directed to a single antigenic site. Whereas polyclonal antibodies bind to different epitopes on one antigen, monoclonal antibodies are directed to a single epitope on one antigen. Monoclonal antibodies have high selectivity and specificity suitable for use in diagnosis and analysis based on antigen-antibody conjugation, and enjoy the advantage of not being contaminated by other immunoglobulins because they are produced by fused cells. Monoclonal antibodies can be produced using well-known techniques, such as a hybridoma method (Kohler and Mislstein (1976) European Journal of Immunology 6:511-519) or a phage antibody library technique (Clackson et al, Nature, 352:624-628, 1991).

[26] In a preferable embodiment of the present invention, mice 6 ~ 8 weeks old were immunized by injecting the hybridoma cell line into the peritoneal cavity. From the ascites fluid thus formed, monoclonal antibodies against *Mycoplasma pneumoniae* were isolated. In brief, the ascites fluid was extracted, added with ammonium sulfate, and centrifuged to isolate antibodies. These were purified through dialysis and then

through column chromatography. The monoclonal antibodies thus obtained were called monoclonal antibody 2G6D7.

[27] This pure antibody, prepared according to the present invention, is useful in the preparation of a diagnostic kit. The diagnostic kit comprises a strip on which a test line (T) for showing a diagnosis result and a control line (C) for determining test validity are provided. Protein A and the anti-adhesin P1 monoclonal antibody are completely immobilized at the test line and the control line, respectively, on a nitrocellulose membrane.

[28]

[29] On the other hand, the purified monoclonal antibody may be applied to the direct detection of an antigen of *Mycoplasma pneumoniae*.

[30] Accordingly, the present invention also provides a composition for the diagnosis of *Mycoplasma pneumoniae* infection, comprising the monoclonal antibody.

[31] In accordance with still a further aspect thereof, the present invention provides a kit for the diagnosis of *Mycoplasma pneumoniae* infection, using the monoclonal antibody produced by the hybridoma cell line described above.

[32] In the diagnostic kit of the present invention, an antigen-antibody conjugate is detected using a color particle agglutination assay. Examples of the color particles include colloidal gold particles and color glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads, with preference for gold colloid.

[33] In the diagnostic kit of the present invention, as mentioned above, a capture is provided for binding to the antigen-antibody conjugate. As the capture, Protein A, Protein G or anti-human IgG may be used. In an embodiment of the present invention, Protein A served as a capture for binding an antigen-antibody conjugate.

[34] Examples of the assay system suitable for use in the diagnostic kit of the present invention include, but are not limited to, an ELISA plate, a dipstick device, an immunochromatography and radioimmunoassay device, and a flow-through device. Preferable is a diagnostic kit in a form of a strip or a device using immunochromatography.

[35] A kit for the diagnosis of Mycoplasma infection using immuno-chromatography in accordance with the present invention is composed essentially of a nitrocellulose membrane, on which two invisible lines are drawn, and a glass-fiber pad in which a recombinant protein-gold conjugate is stored in a dry state. At the positions of the two invisible lines, respective different proteins are immobilized on the nitrocellulose membrane. Protein A is immobilized at a lower test line (T), while an anti-adhesin P1 monoclonal antibody is immobilized at a lower control line (C). In the glass fiber pad, an antigen-gold conjugate, made of the recombinant adhesion P1 protein and gold particles, is absorbed and dried.

[36] When a specimen is loaded onto a sample window of the kit, the dry antigen-gold conjugate, stored in the glass fiber pad, is hydrated and bound to the antibody of the specimen, with concomitant migration to the nitrocellulose membrane through micropores by capillary action. Subsequently, as the Protein A, immobilized at the invisible line, is associated with the antibody bound to the antigen-gold conjugate, the line turned red because of the red color of the antigen-gold conjugate. As for the anti-adhesin P1 monoclonal antibody immobilized at the upper control line, it always reacts with an unbound antigen-gold conjugate, even if it passes without reaction to the antibody of the specimen or cannot bind to the antibody because it is absent in the specimen. Therefore, the control line appears red in every test, indicating whether the test has been conducted properly or not. In other words, when an antibody to Mycoplasma is present in the specimen, the test line and the control line appear red simultaneously. By contrast, when the specimen contains no antibodies to Mycoplasma, the control line is visible while the test line remains invisible.

[37] In accordance with a modification of the embodiment, the present invention can determine whether the human antibody to Mycoplasma is IgG or IgM. In greater detail, a kit is constructed in such a manner that anti-human IgG, anti-human IgM and an anti-adhesin P1 monoclonal antibody are immobilized at line 1, line 2 and a control line, respectively. When a specimen is developed on the glass fiber pad, the anti-adhesin P1 antibody of the specimen is bound to the antigen-gold conjugate and then reacted at line 1 when the antibody is IgG or at line 2 when the antibody is IgM. As a result, when the specimen contains IgG against adhesin P1, line 1 appears red, while when the specimen contains IgM against adhesin P1, line 2 appears red.

[38]

[39] The diagnostic kit of the present invention is found to show 90% or higher sensitivity and specificity for Mycoplasma pneumonia compared to commercially available kits (e.g., SeroDia Myco II kit, Fujirebio, Japan). It can diagnose mycoplasma pneumonia more rapidly and conveniently than can conventional kits (see Example 7). Accordingly, the diagnostic kit of the present invention can be conveniently and easily applied to the diagnosis of pulmonary paragonimiasis.

[40]

[41] In accordance with still another aspect thereof, the present invention provides a method for diagnosing mycoplasma infection using the diagnostic kit described above.

[42] In detail, a biological specimen (e.g., serum or plasma) suspected to be infected with *Mycoplasma pneumoniae* is loaded onto the sample window of the diagnostic kit of the present invention, and after the completion of reaction, a decision is made about the infection of *Mycoplasma pneumoniae* with reference to the appearance of the lines of the kit.

[43]

[44] As used herein, the term "biological specimen", is intended to refer to a blood sample(e.g., serum or plasma) from mammals suspected of being infected with pulmonary paragonimiasis, or to be tested therefor, including humans. For convenience, it is also expressed just as "specimen" or "sample". A biological specimen may or may not be diluted before being brought into contact with the sample window of the diagnostic kit according to the present invention.

Advantageous Effects

[45] The diagnostic kit of the present invention can be more effectively used to diagnose *Mycoplasma pneumoniae* infection than can conventional techniques such as gelatin particle agglutination assay and ELISA because it takes a shorter time period using the diagnostic kit. In addition, the present invention promises an improvement in therapeutic effect because it significantly reduces misdiagnosis rates and thus prevents the misuse and overuse of antibiotics.

[46]

Brief Description of the Drawings

[47] FIG. 1 is a schematic view illustrating the membrane-associated proteins of *Mycoplasma pneumoniae*.

[48] FIG. 2 is a flow chart showing processes of preparing a recombinant protein of the adhesin P1 of *Mycoplasma pneumoniae*.

[49] FIG. 3 is a photograph of SDS-PAGE (SDS-polyacrylamide gel electrophoresis) showing the purification of the recombinant adhesin P1 of the present invention, in which a cell lysate obtained after protein expression (lane 1), a cell lysate obtained before protein expression(lane 2), and a purified recombinant adhesin P1 (lane 3) are run, along with a marker (lane M).

[50] FIG. 4 shows the structure of a strip-type kit for diagnosing *Mycoplasma pneumoniae* infection based on rapid immunochromatography, in schematic diagrams.

[51] FIG. 5 shows positive and negative diagnosis results from a strip-type kit for diagnosing *Mycoplasma pneumoniae* infection based on rapid immunochromatography in photographs.

[52] FIG. 6 shows the structure of a device-type kit for diagnosing *Mycoplasma pneumoniae* infection based on rapid immunochromatography, in schematic diagrams.

[53] FIG. 7 shows positive and negative diagnosis results of a device-type kit for diagnosing *Mycoplasma pneumoniae* infection based on rapid immunochromatography, in photographs.

[54]

Mode for the Invention

[55] A better understanding of the present invention may be obtained through the following examples which are set forth to illustrate, but are not to be construed as the limit of the present invention.

[56]

[57] EXAMPLE 1: Gene Cloning, Expression and Purification of Adhesion P1 of *Mycoplasma pneumoniae*

[58]

[59] 1-1. Gene Cloning of Adhesin P1 of *Mycoplasma pneumoniae*

[60] The genomic DNA of *Mycoplasma pneumoniae* was purchased from Advanced Biotechnologies Inc, U.S.A. A fragment of the adhesion P1 gene, 1053 bp in length, represented by SEQ ID NO. 2, was amplified with the genomic DNA serving as a template. That is, the DNA fragment was selected for the reason that when it was expressed, the resulting recombinant protein was predicted 1) not to be in a truncated form due to the lack of UGA sequences therein, 2) contain many epitopes with high antigenicity, as simulated by epitope prediction software, and 3) be water-soluble with a pI less than 7 and an average hydrophobicity index less than zero. PCR was performed using a pair of primers AP1F (SEQ ID NO. 3) and AP1R (SEQ ID NO. 4) to amplify a 1053 bp-long DNA fragment corresponding to a region of the adhesin P1 gene. The PCR product thus obtained was cloned to a pGEM-Easy T vector (Promega, U. S. A.). The clone was analyzed for base sequence by Bioneer Corporation, Korea, and was found to be incident with a 1053 bp-long, initial adhesion P gene sequence. Comparison with the base sequence of *Mycoplasma pneumoniae* searched with the NCBI GenBank database (GenBank Accession Number: AF290001) indicated that the amplified clone is part of the adhesion P1 gene of *Mycoplasma pneumoniae*. Thereafter, the DNA fragment was subcloned to a pGEX-4T-1 expression vector (GE Healthcare, Sweden) after being digested with EcoRI in the 5' direction and with XhoI in the 3' direction. The expression of the gene was performed in E. coli BL21 (DE3).

[61]

[62] 1-2. Bacterial Expression and Purification of Recombinant Adhesin P1 Protein

[63] The E. coli BL21 (DE3) carrying the recombinant pGEX-4T-1-P1 expression vector was cultured to an OD of 0.6 ~ 0.8 at 600 nm. After treatment with 1 mM of IPTG (isopropyl-β-D-thiogalactopyranoside), culturing for additional 4 ~ 5 hrs induced the overexpression of the recombinant adhesin P1 protein. The E. coli cells were collected by centrifugation and lysed using an ultrasonicator. After the centrifugation of the cell lysate, the supernatant was subjected to affinity chromatography using GSH-bound Sepharose. In this regard, the supernatant was loaded on a column with GSH-bound Sepharose to attach GST-adhesin P1 fusion protein to the Sepharose, followed by washing off unbound materials with a buffer. Thereafter, the GST-adhesin P1 fusion

protein was eluted from the column with a Tris buffer (pH 7.0) containing 100 mM of GSH (glutathione). The eluted fractions were dialyzed to concentrate the GST-adhesin P1 fusion protein, which was then used in the present invention without additional treatment. If necessary, GST was removed by treatment with thrombin (GE healthcare, Sweden) to isolate only the recombinant adhesion P1 protein before use in the present invention.

[64]

[65] EXAMPLE 2: Production of Monoclonal Antibody for Recombinant Adhesin P1

[66]

[67] 2-1. Immunization with the Recombinant Adhesin P1 Protein

[68] A solution of 100 μg of the recombinant adhesin P1, protein prepared in Example 1, was injected, along with an equal volume of a complete Freund's adjuvant suspension, into the peritoneal cavity of a female mouse 6 ~ 8 weeks old (BALB/C, Dae Han BioLink Co. Ltd., Korea). An incomplete Freund's adjuvant was injected once into the mouse two weeks later and once more an additional two weeks later. Two days after the final immunization, a serum sample was taken from the tail of the mouse, 1/1000 diluted in PBS and analyzed for antibody titer using ELISA (enzyme-linked immunosorbent assay). If the antibody titer was low, immunization was again performed two weeks later.

[69]

[70] 2-2. Fusion of Splenocyte with Myeloma Cells

[71] The spleen was excised from the mouse immunized in the same manner as in Example 2-1 and homogenized using a tissue homogenizer. The cell suspension was centrifuged to recover splenocytes which were then counted. Myeloma cells were removed from a cell culture flask, suspended in an RPMI 1640 medium and counted. 1×10^7 myeloma cells and 1×10^8 splenocytes were mixed and suspended in an RPMI 1640 medium in a 50 ml flask, followed by centrifugation at 200×g for 5 min to recover the cells. These cells were resuspended in an RPMI 1640 medium and added with 1 ml of a PEG solution (50% polyethylene glycol) over 1 min with slow shaking. 5 min later, an RPMI 1640 medium was slowly added in an amount of 1 ml over 30 sec, again in an amount of 3 ml over 30 sec, again in an amount of 17 ml over 1 min, and finally in an amount of 20 ml. The cells were allowed to stand for 5 min. After centrifugation at 200×g for 5 min, the medium was removed and the cells were carefully suspended in 50 ml of an RPMI1640 1% HAT (hypoxathine-aminopterin-thymidine) and 100 μl of the cell suspension was layered on feeder cells in each well of 96-well plates before incubation at 37℃ in a 5% CO_2 incubator.

[72]

[73] 2-3. Cloning of Fused Cell Line and Antibody Production Using Ascites Fluid

[74] When the fused cells of Example 2-2 gave rise to colonies during incubation for 10 days, 100 μl of the medium was taken from each well and assayed for antibody activity against adhesion P1 through ELISA. After each test for antibody activity, the cells, which were identified to produce the antibodies, were transferred to 24-well cell culture plates and incubated. The cloning procedure was repeated until stable monoclonal cell lines were obtained. The cell lines were cultured in T flasks. As soon as enough cells were present, they were harvested, frozen and stored in liquid nitrogen.

[75]

[76] 0.5 ml of an incomplete Freund's adjuvant was injected into the peritoneal cavity of a mouse (BALB/C) 6 ~ 8 weeks old. On Day 7 after the injection, the fused cells were suspended at a density of 1.5×10^6 cells in 0.5 ml of PBS, and this cell suspension was peritoneally injected into the mouse. After 1 ~ 2 weeks, the ascites fluid was sampled with a syringe and freeze-stored.

[77]

[78] 2-4. Purification of Monoclonal Antibody

[79] Ammonium sulfate was added at a concentration of 10% to the ascites fluid of Example 2-3 and mixed for 30 min. After centrifugation at 15,000×g for 30 min, ammonium sulfate was added to the supernatant at a concentration of 50% and the mixture was left at 4℃ for 30 min. After an additional centrifugation at 15,000×g for 30 min, the pellet was suspended in a 20 mM phosphate buffer solution (pH 7.0). The suspension was dialyzed in 20 mM PBS for 18 hrs or longer and loaded on a protein G-coupled column equilibrated with 20 mM PBS (pH 7.0), followed by washing with PBS to remove unattached materials. The antibodies bound to the column were eluted with a 10 mM glycine solution (pH 2.8). In this regard, 1 M Tris (pH 9.0) was added at a 1/10 volume of the eluent so as to adjust the pH to 7.0 ~ 7.5. After dialysis in 150 mM PBS, the antibody fraction was quantified for antibody activity using a Bradford assay and frozen and stored until use. The purified monoclonal antibodies were named monoclonal antibody 2G6D7.

[80]

[81] EXAMPLE 3: Construction of Strip-Type Diagnostic Kit Using Rapid ImmunoChromatography Assay with Recombinant Antigen

[82]

[83] 3-1. Immobilization of Protein A and Anti-Adhesin P1 Monoclonal Antibody

[84] Protein A and anti-adhesin P1 monoclonal antibody 2G6D7 were allocated at respective specific positions, that is, a test line (T) and control line (C), on a nitrocellulose membrane layered on a plastic card, followed by incubation under dry conditions at 30℃ for 2 days in an incubator to completely immobilize the protein A

and the monoclonal antibody onto the membrane.

[85]

[86] 3-2. Preparation of Gold Conjugate and Gold Conjugate Pad

[87] The recombinant adhesin P1 protein was incubated along with colloidal gold particles 40 nm in size in a 37°C water bath for 1 hr to associate them with each other. Thereafter, BSA (bovine serum albumin) and sucrose were added at concentrations of 3% and 1%, respectively, followed by incubation for an additional 1 hr so as to associate them with the colloidal gold particles which remained unbound. The recombinant protein-gold conjugates were harvested by centrifugation at 10,000×g for 20 min and analyzed for absorbance at 540 nm before cold storage. The gold conjugate was infused into a plastic microwell plate and dried to prepare a gold conjugate well.

[88]

[89] 3-3. Assembly of Strip Kit

[90] As illustrated in FIG. 4, a strip-type plastic card was overlaid with the protein A and monoclonal antibody-immobilized nitrocellulose membrane for a half region thereof while an absorption pad capable of absorbing a specimen and a buffer was attached onto the other half region of the plastic card to prepare a strip-type kit.

[91]

[92] EXAMPLE 4: Construction of Device-Type Diagnostic Kit Using Rapid ImmunoChromatography with Recombinant Antigen

[93]

[94] 4-1. Immobilization of Protein A and Anti-Adhesin P1 Monoclonal Antibody

[95] Protein A and anti-adhesin P1 monoclonal antibody 2G6D7 were allocated at respective specific positions, that is, a test line (T) and a control line (C), on a nitrocellulose membrane layered on a plastic card, followed by incubation under dry conditions at 30°C for 2 days in an incubator to completely immobilize the protein A and the monoclonal antibody on the membrane, as shown in FIG. 4.

[96]

[97] 4-2. Preparation of Gold Conjugate and Gold Conjugate Pad

[98] The recombinant adhesin P1 protein was incubated along with colloidal gold particles 40 nm in size in a 37°C water bath for 1 hr to associate them with each other. Thereafter, BSA (bovine serum albumin) and sucrose were added at concentrations of 3% and 1%, respectively, followed by incubation for an additional 1 hr so as to associate them with the colloidal gold particles that remained unbound. The recombinant protein-gold conjugates were harvested by centrifugation at 10,000×g for 20 min and analyzed for absorbance at 540 nm before cold storage. The gold conjugate was infused into a glass fiber pad and dried to prepare a gold conjugate pad.

[99]

[100] 4-3. Assembly of Device-Type Kit

[101] As shown in FIG. 6, the nitrocellulose membrane, immobilized with protein A and the monoclonal antibody, was attached onto a mid-region of the plastic card while the opposite end regions of the plastic card were overlaid respectively with an absorption pad, and a sample pad serving to load a sample thereon. This strip construct was assembled with a plastic housing, as shown in FIG. 6, to prepare a diagnostic kit.

[102]

[103] EXAMPLE 5: Diagnosis with Strip-Type Kit

[104] One drop of a washing buffer was placed in the gold conjugate well provided along with the kit. This was mixed with four microliters of a blood sample using a pipette. The absorption pad attached to the strip-type kit was put into the gold conjugate well for 10 min so as to absorb the sample solution sufficiently, and then into a washing well containing three drops of an assay buffer for 10 min so as to wash the membrane. Infection with *Mycoplasma pneumoniae* could be read with reference to the positions at which red lines appear, as illustrated in FIG. 5.

[105]

[106] EXAMPLE 6: Diagnosis with Device-Type Kit

[107] A device-type kit was unpacked from an aluminum pouch and placed on a flat surface. Four microliters of a blood sample was loaded on a sample window, as shown in FIG. 6a, so that it was absorbed by the absorption pad. Thereafter, three drops of an assay buffer were loaded. 15 min later, the presence of the antibody to *Mycoplasma pneumoniae* could be read according to the red appearance of the test line.

[108]

[109] EXAMPLE 7: Efficacy Test of Strip- and Device-Type Diagnostic Kits Using Rapid Immunochromatography

[110]

[111] 7-1. Efficacy Test for Positive Sensitivity

[112] The diagnostic kits of Examples 3 and 4 were tested for sensitivity for Mycoplasma antibody-positive clinical specimen. This test was conducted with 95 positive clinical samples provided by Chungju St. Mary's Hospital, Korea. For comparison, the same samples were tested with SeroDia Myco II Kit (Fujirebio, Japan). The results are summarized in Table 1, below.

[113]

[114] Table 1

[Table 1]

95 Positive Samples		Serodia Mycoll		
		Positive	Negative	
Inventive Kit	Positive	89	1	90
	Negative	5	0	5
Sum		94	1	95
[1]Sensitivity of the Inventive Kit		94.7% (90/95)		
[1]Sensitivity of Serodia Mycoll		98.9% (94/95)		

$$[1]\text{Sensitivity} = \frac{\text{No.of Positively Reacted Kits}}{\text{No.of Tested Positive Samples}} \times 100(\%)$$

[115]

[116] 7-2. Efficacy Test for Negative Selectivity

[117] The diagnostic kits of Examples 3 and 4 were tested for specificity for Mycoplasma antibody-negative clinical specimens. This test was conducted with 120 negative clinical samples provided by the Parasitology Lab of Medical College, SungKyunKwan University, Korea. For comparison, the same samples were tested with SeroDia Myco II Kit (Fujirebio, Japan). The results are summarized in Table 2, below.

[118]

[119] Table 2

[Table 2]

120 Negative Samples		Serodia Mycoll		
		Positive	Negative	
Inventive Kit	Positive	1	0	1
	Negative	10	109	119
Sum		11	109	120
[1]Sensitivity of the Inventive Kit		99.1% (119/120)		
[1]Sensitivity of Serodia Mycoll		90.8% (109/120)		

$$[1]\text{Specificity} = \frac{\text{No.of Negatively Reacted Kits}}{\text{No.of Tested Negative Samples}} \times 100(\%)$$

[120]

[121] 7-3. Conclusion

[122] As described hitherto, the diagnostic kits of the present invention are more convenient for use and allow more rapid diagnosis without the aid of expensive in-

struments, compared to conventional diagnostic kits. In addition to these advantages, the diagnostic kits of the present invention are of high selectivity and specificity, and thus show clinically highly useful applicability.

[123]

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : CHUNG, Chan-Bok
Bioland
644-6 Gek-ri, Ochang-myun, Cheongwon-gun, Chungbuk 363-883
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: **2G6D7** **(hybridoma cell line)**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 11242BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [x] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **November 15, 2007.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korean Collection for Type Cultures** Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s): OH, Hee-Mock, Director Date: **November 29, 2007**

Form BP/4 (KCTC Form 17) sole page

Claims

[1] A composition for use in the diagnosis of *Mycoplasma pneumoniae* infection, comprising a monoclonal antibody specific for a recombinant adhesin P1 protein of *Mycoplasma pneumoniae*.

[2] The composition according to claim 1, wherein the recombinant adhesin P1 protein of *Mycoplasma pneumoniae* has an amino acid sequence of SEQ ID NO. 1.

[3] The composition according to claim 1, wherein the monoclonal antibody specific for a recombinant adhesin P1 protein of *Mycoplasma pneumoniae* is monoclonal antibody 2G6D7.

[4] The composition according to claim 3, wherein the monoclonal 2G6D7 is produced by a fused cell of Deposition No. KCTC 11242BP.

[5] A diagnostic kit for *Mycoplasma pneumoniae* infection, comprising the composition of one of claims 1 to 4.

[6] The diagnostic kit according to claim 5, which is in a form of a strip type using immunochromatography for diagnosis.

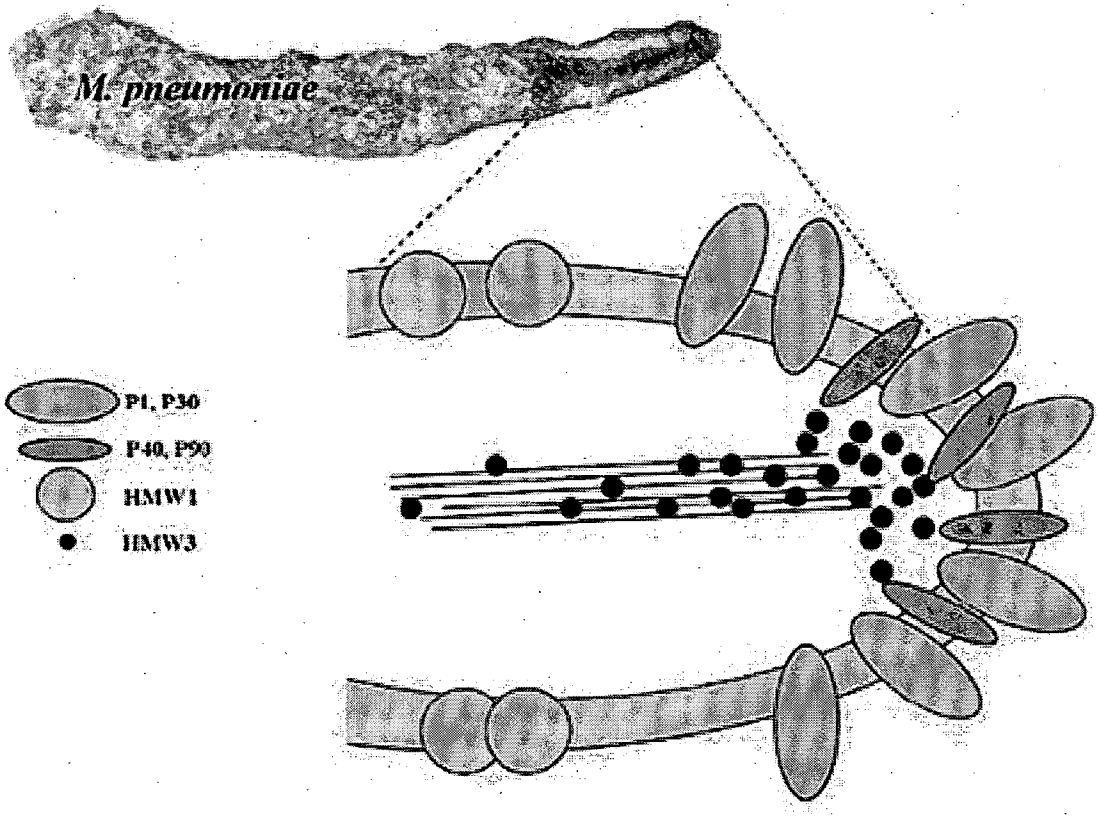
[7] The diagnostic kit according to claim 5, which is in a form of a device type using immunochromatography for diagnosis.

[8] The diagnostic kit according to claim 5, which uses a color particle agglutination assay in detecting an antibody-antigen conjugate.

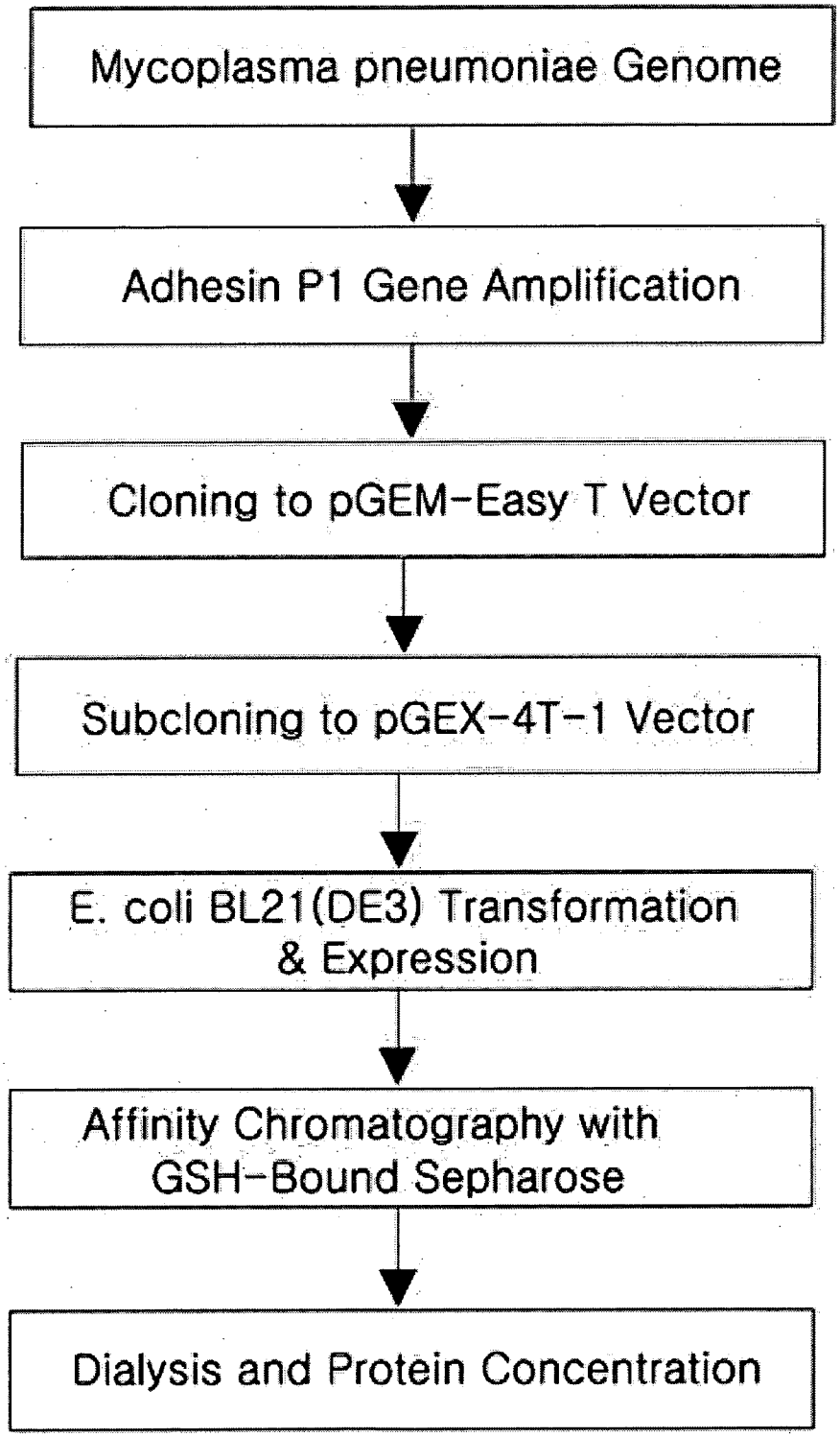
[9] Antibody 2G6D7, produced by a fused cell of Deposition No. KCTC 11242BP.

[10] A fused cell of Deposition No. KCTC 11242BP.

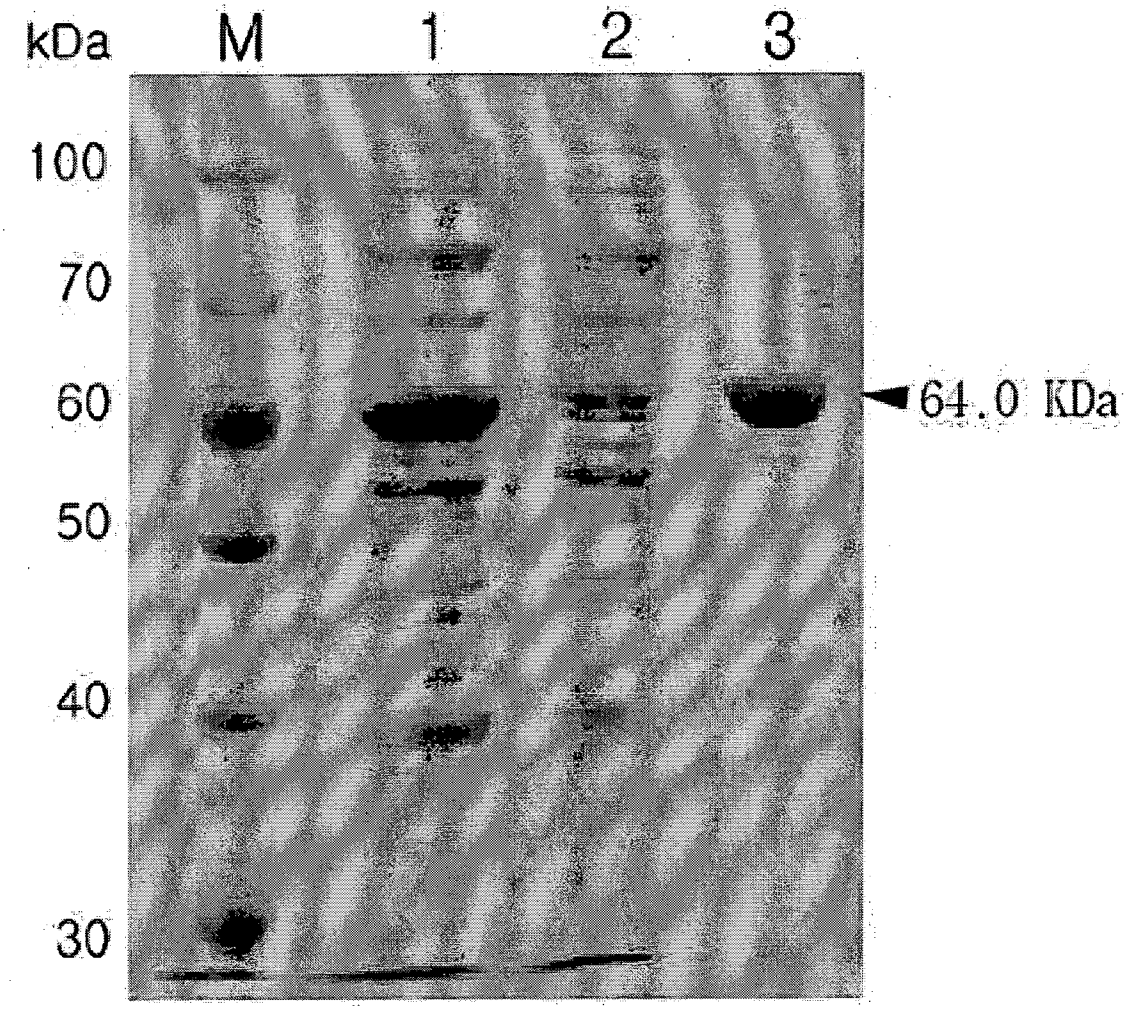
[Fig. 1]



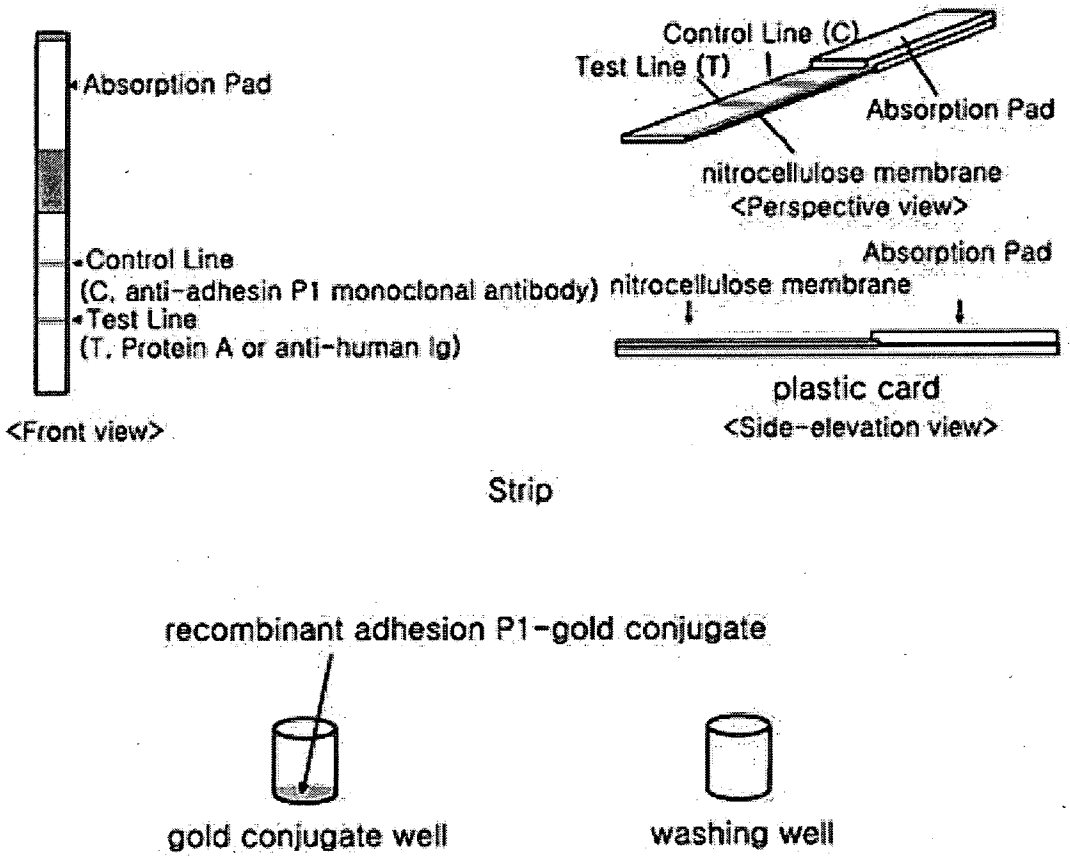
[Fig. 2]


Mycoplasma pneumoniae Genome
Adhesin P1 Gene Amplification
Cloning to pGEM-Easy T Vector
Subcloning to pGEX-4T-1 Vector
E. coli BL21(DE3) Transformation & Expression
Affinity Chromatography with GSH-Bound Sepharose
Dialysis and Protein Concentration

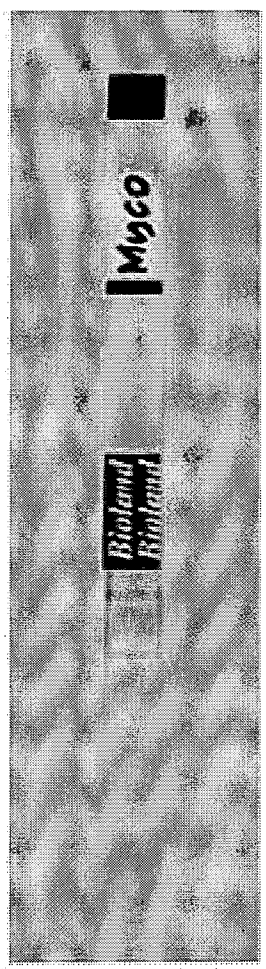
[Fig. 3]



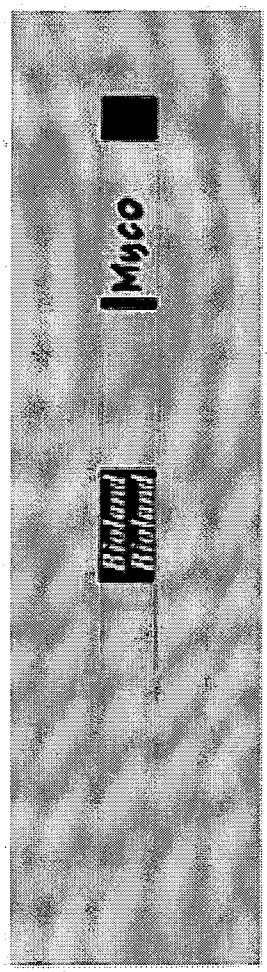
[Fig. 4]


Absorption Pad
Control Line
(C, anti-adhesin P1 monoclonal antibody)
Test Line
(T, Protein A or anti-human Ig)
<Front view>
Control Line (C)
Test Line (T)
Absorption Pad
nitrocellulose membrane
<Perspective view>
Absorption Pad
nitrocellulose membrane
plastic card
<Side-elevation view>
Strip
recombinant adhesion P1-gold conjugate
gold conjugate well
washing well

[Fig. 5]

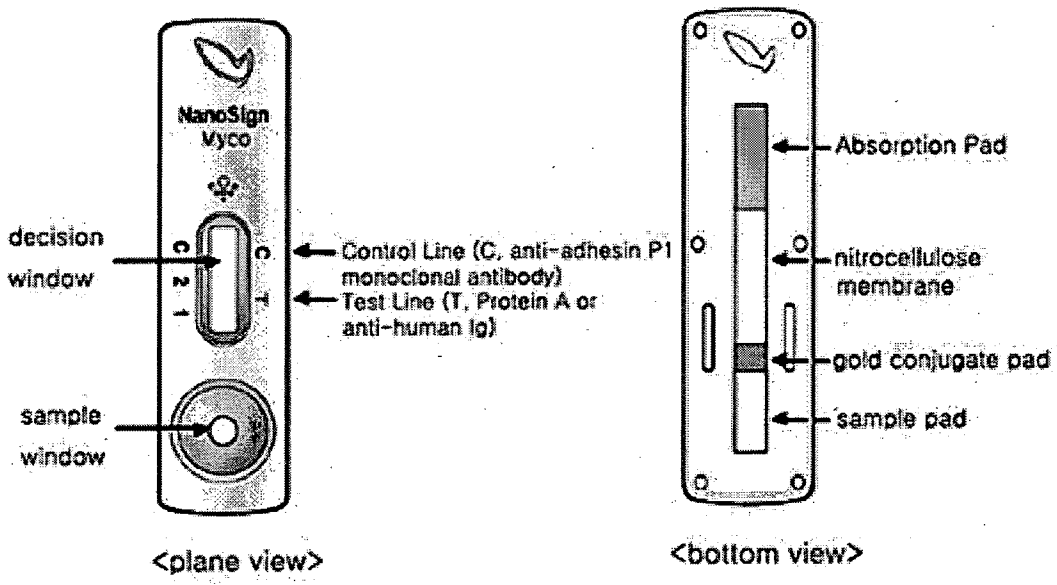


Positive example

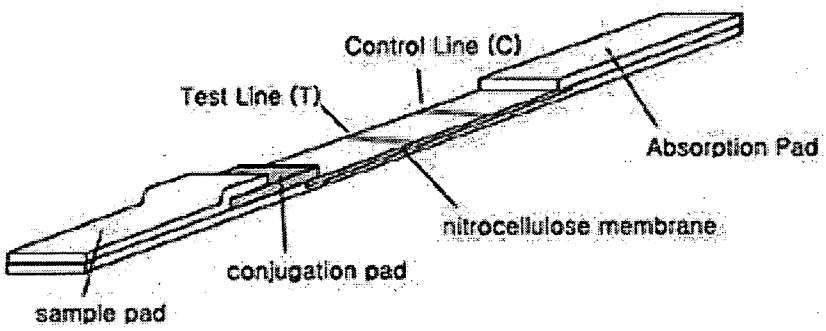


negative example

[Fig. 6]

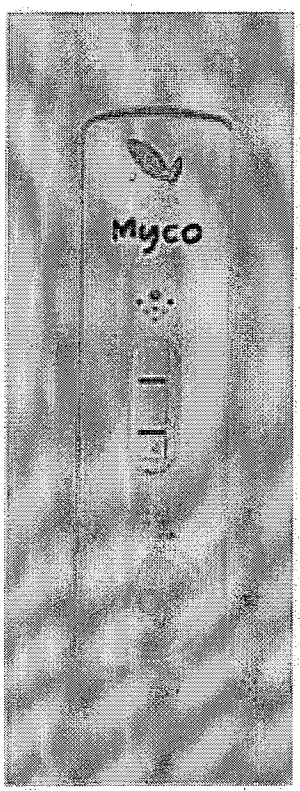


<plane view> <bottom view>

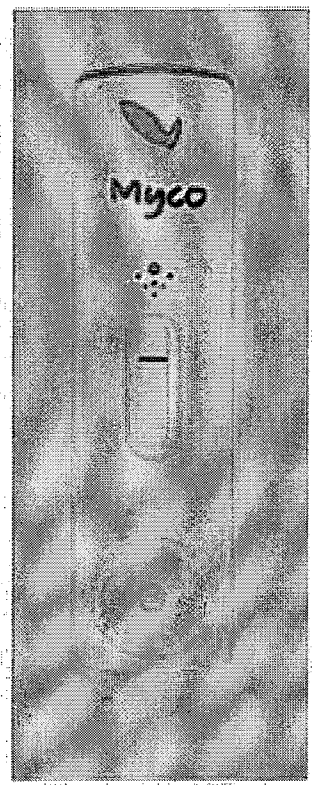


<Strip for device-type kit>

[Fig. 7]



<Positive example>



<Negative example>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/003092

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/569(2006.01)i, G01N 33/68(2006.01)i, G01N 33/53(2006.01)i, G01N 33/531(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 G01N33/569

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, NCBI PubMed database, Delphion Research Intellectural Property Network database, google scholar, BLAST database "adherin P1", "Mycobacteria pneumoniae", " diagnosis", "antibody"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- A	JP 05-304990 A (BEHRINGWERKE AKTIENGESELLSCHAFT) 19 November 1993 See the abstract and claims.	1,5-8 ---------- 2-4,9,10
A	US 4,945,041 A (BASEMAN J. et al.) 31 July 1990 See the abstract.	1-10
A	WO 92/001808 A1 (DOJIN IYAKU-KAKO CO., LTD. et al.) 06 February 1992 See the abstract and claims.	1-10
A	KR 10-2002-0073192 A (ASAHI KASEI KABUSHIKI KAISHA) 19 September 2002 See the claims and examples.	1-10

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent 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 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 when the document is taken alone

"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

Date of the actual completion of the international search	Date of mailing of the international search report
28 NOVEMBER 2008 (28.11.2008)	**28 NOVEMBER 2008 (28.11.2008)**

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

Sohn, Younghee

Telephone No. 82-42-481-5975

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/003092

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

   a. type of material
   - [x] a sequence listing
   - [ ] table(s) related to the sequence listing

   b. format of material
   - [ ] on paper
   - [x] in electronic form

   c. time of filing/furnishing
   - [ ] contained in the international application as filed
   - [x] filed together with the international application in electronic form
   - [ ] furnished subsequently to this Authority for the purposes of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/003092

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 05-304990 A	19.11.1993	AU 2712192 A	22.04.1993
		AU 666134 B2	01.02.1996
		CA 2080713 A1	18.04.1993
		DE 4134297 A1	22.04.1993
		EP 0537497 A2	21.04.1993
		US 5641638	24.06.1997
		US 5641638 A	24.06.1997
US 4,945,041 A	31.07.1990		
		US 5369005	29.11.1994
		CA 1319335 C	22.06.1993
WO 92-01808 A1	06.02.1992		
		KR 10-1993-0700674	15.03.1993
KR 10-2002-0073192 A	19.09.2002	AU 2001-30532 A1	14.08.2001
		AU 3053201 A	14.08.2001
		CA 2398469 A1	09.08.2001
		CN 1406279 A	26.03.2003
		CN 1406279	26.03.2003
		WO 01-057199	09.08.2001
		TW 282368 B	11.06.2007
		US 2004-0014943 A1	22.01.2004
		WO 01-57199 A1	09.08.2001