DESCRIPTION

Anti-Influenza A Virus Monoclonal Antibody and Immunoassay Instrument Using the Antibody

Technical Field

The present invention relates to an anti-influenza A virus monoclonal antibody and immunoassay device using the same.

Background Art

Influenza periodically repeated worldwide prevalence from old times and a number of people died of the disease in each time of the prevalence. In 1931, the causative virus was detected and a road to elucidation of the cause was opened up. The causative virus of influenza is classified into three types, that is, types A, B and C, depending on the antigenicity of the soluble nucleoprotein (NP) located in the virus. Influenza A virus is further classified into subtypes depending on the antigenicities of two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA).

Since 1972, for prophylaxis of influenza, a vaccine inactivated by formalin after ether treatment has been used. In recent years, in addition to the vaccine used for the prophylaxis, anti-influenza drugs were discovered and are now widely used. These therapeutic drugs include amantadine hydrochloride effective for influenza A virus, and zanamivir and oseltamivir phosphate effective for influenza A and B viruses.

In the selection of such a therapeutic drug, it is important to detect an influenza virus in the sample, to confirm that the cause of infection is the influenza virus, and to determine whether the type of influenza virus is A or B. Further, since influenza A virus is more infectious than influenza B virus and causes serious symptoms, determination of the type of the infected virus is especially important for early therapy.

Influenza viruses have been conventionally detected by using anti-influenza virus antibodies. As for antibodies to influenza A virus, in order to help the production of vaccine after determining the subtype of the virus expected to prevail, antibodies for determining the subtype of the virus, which specifically recognize HA or NA of the virus, are known (see, for example, Patent Literatures 1 and 2). The amino acid sequences of the nucleoproteins of the subtypes of influenza A virus have been determined and reported in National Library of Medicine; Entrez Nucleotides database and the like.

As a device for detecting influenza virus, a flow through type device is known (see Patent Literature 3), which comprises a solid phase on which an influenza virus antigen may be immobilized. With this device, the influenza virus in a sample is reacted with the solid phase, then an anti-influenza A virus antibody labeled with a first enzyme and an anti-influenza B virus antibody labeled with a second enzyme are reacted with the solid phase, then substrates are added to carry out the reaction, and the coloring of the solid phase is visually observed. Although this device utilizes antibodies to nucleoproteins of influenza viruses, the measurement operations are complicated, so that the device is not a simple measuring device. Further, an immunoassay device (hereinafter referred to as "immunochromatography device") utilizing a monoclonal antibody to the nucleoprotein of influenza virus is known (see Non-patent Literature 1).

On the other hand, an immunochromatography device using a belt-like matrix through which liquid can be transported was developed. By virtue of this device, an antibody or antigen in a sample can be measured simply in a short time by only spotting a sample on the prescribed area, without a special device or a skillful measuring technique. For example, an immunochromatography device has been developed, in which a plurality of *Treponema pallidum* antigens (TP antigens) are bound to a detection zone, and a plurality of anti-TP antibodies in a sample may be

detected in separate detection zones (see Patent Literature 4). This device comprises a plurality of detection zones on which different TP antigens are bound, respectively. With this device, different anti-TP antibodies are detected separately in one measurement, thereby the time of infection is determined, which is used for the selection of the therapeutic drug or the like.

Patent Literature 1: Japanese Laid-open Patent Application (Kokai) No. 6-100594

Patent Literature 2: Japanese Laid-open Patent Application (Kokai) No. 7-304799

Patent Literature 3: Japanese Laid-open Patent Application (Kokai) No. 2001-124775

Patent Literature 4: Japanese Laid-open Patent Application (Kokai) No. 9-229938

Non-patent Literature 1: The Journal of the Japanese Association for Infectious Diseases, 2001;75: 792-799

Disclosure of the Invention

Problems Which the Invention Tries to Solve

Although antibodies to nucleoprotein of influenza A virus have been conventionally used in immunochromatography methods or the like, the known antibodies have poor reactivity and specificity to influenza A virus, so that they are not satisfactory for the highly sensitive immunoassays. Further, for the selection of the therapeutic drug for influenza, an antibody which does not react with influenza B virus but reacts with all influenza A viruses including subtypes in which HA and NA are mutated, is demanded.

Further, a device is demanded, by which distinguishment between influenza A virus and influenza B virus may be attained in one operation for a short time without using a special diagnosis equipment or a measuring device.

Accordingly, an object of the present invention is to provide an anti-influenza A virus antibody having a high specificity. Another object of the present invention is to provide an immunoassay device by which influenza A virus may be specifically

detected.

Means for Solving the Problems

The present inventors intensively studied to succeed in creating an anti-influenza A virus antibody corresponding to the nucleoprotein of influenza A virus, which specifically reacts with influenza A virus, thereby completing the present invention. The present inventors also inferred that an immunoassay device may be provided, with which influenza A virus may be detected in a manner distinguishing from influenza B virus, by utilizing this anti-influenza A virus antibody.

That is, the present invention provides an anti-influenza A virus monoclonal antibody which undergoes antigen-antibody reaction with a nucleoprotein having a molecular weight of 55 kD to 70 kD of influenza A virus and which does not substantially undergo antigen-antibody reaction with influenza B virus, or an antigen-binding fragment thereof. The present invention also provides an immunoassay device comprising a labeled reagent zone having a labeled mobile anti-influenza A virus antibody, a sample-spotting zone, a developer-supply zone, developer-absorption zone and an influenza A virus-detection zone in which an anti-influenza A virus antibody is immobilized on a matrix, which zones are provided in the matrix, wherein at least one of the labeled anti-influenza A virus antibody and the anti-influenza A virus antibody immobilized on the detection zone is the above-described monoclonal antibody according to the present invention.

Effect of the Invention

By the present invention, an anti-influenza A virus monoclonal antibody which immunologically reacts with influenza A virus but does not react with influenza B virus was provided. By immunoassays using the anti-influenza A virus monoclonal antibody according to the present invention, influenza A virus may be detected or quantified in a manner distinguishing from influenza B virus. By the present invention, an immunoassay device using the above-described anti-influenza

A virus monoclonal antibody according to the present invention was also provided. By using the immunoassay device according to the present invention, influenza A virus may be detected simply and in a manner distinguishing from influenza B virus. Therefore, it is expected that the present invention will greatly contribute to the diagnosis and therapy of influenza.

Brief Description of the Drawings

Fig. 1 shows the results of the experiments in which the monoclonal antibodies according to the present invention were reacted with the antigens fractioned by Western blot method.

Fig. 2 shows the restriction map of the expression plasmid (pW6A) used in Example 4.

Fig. 3 shows amino acid numbers of fragment A1 to A7 obtained by fragmentation of the A fragment of influenza H1N1.

Fig. 4 shows the CBB staining pattern and the results of Western blot, for confirming the reactivity with FVA2-11.

Fig. 5 shows the amino acid sequences (80-140) of nucleoproteins of subtypes of influenza A virus.

Fig. 6 is a schematic cross-sectional view of an embodiment of the measuring device according to the present invention.

Fig. 7 is a plan view showing an example of the state wherein the measuring device according to the present invention is set in a cassette.

Fig. 8 is a cross-sectional view taken along the line A-A' in Fig. 7.

Best Mode for Carrying out the Invention

As described above, the monoclonal antibody according to the present invention undergoes antigen-antibody reaction with the nucleoprotein of influenza A virus, which nucleoprotein has a molecular weight of 55 kD to 70 kD. The fact that an antibody undergoes antigen-antibody reaction with the nucleoprotein of influenza

A virus, having a molecular weight of 55 kD to 70 kD may be confirmed by Western blot method involving sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using influenza A virus as the sample. In the Western blot (see Examples below), the monoclonal antibody according to the present invention recognizes the nucleoprotein having a molecular weight of 55 kD to 70 kD.

As mentioned above, the monoclonal antibody according to the present invention does not substantially undergo antigen-antibody reaction with influenza B virus. The term "does not substantially undergo antigen-antibody reaction" herein means that the antibody does not undergo antigen-antibody reaction at a detectable level, or even if it undergoes antigen-antibody reaction, the degree of the reaction is apparently weaker than that of the reaction with influenza A virus so that it is clear for those skilled in the art that the antibody does not undergo antigen-antibody reaction with influenza B virus. The term "does not substantially undergo antigen-antibody reaction with influenza B virus" herein means that the antibody does not substantially undergo antigen-antibody reaction not only with the nucleoprotein of influenza B virus, but also proteins of other element of influenza B virus. In a preferred mode, the monoclonal antibody according to the present invention also does not substantially react with influenza C virus.

In a preferred mode, the monoclonal antibody according to the present invention reacts with an epitope existing in the region between the 59th amino acid (hereinafter indicated as "59aa") and 130aa of the nucleoprotein of influenza A virus. The amino acid sequence of influenza A virus is known and described in, for example, GenBank Accession No. ITY238021 and the like. The amino acid sequences of the region of 59aa-140aa of the nucleoproteins of various subtypes of influenza A virus are shown in Fig. 5. In a preferred mode, since the monoclonal antibody according to the present invention undergoes antigen-antibody reaction with the nucleoprotein of the influenza A virus of each of the subtypes, the monoclonal

antibody is one which reacts with an epitope within the region having a common sequence and having a hydrophilic structure, e.g., the region 90aa-95aa, 111aa-115aa, 120aa-123aa or the like, in the amino acid sequences shown in Fig. 5, or which reacts with an epitope containing these regions and having a common sequence.

In a preferred mode, the monoclonal antibody according to the present invention undergoes antigen-antibody reaction with the nucleoprotein of each subtype of influenza A virus. That is, influenza A virus has hemagglutinin (H) and neuraminidase (N) on the surface of the virus particle, and is classified into various subtypes depending on the differences in the structures of H and N. The monoclonal antibody according to the present invention preferably undergoes antigen-antibody reaction with the nucleoproteins of at least the following subtypes, and more preferably, with the nucleoproteins of all subtypes of influenza A virus. H1N1, H2N2, H3N2, H3N8, H4N6, H5N2, H6N2, H7N7, H8N4, H9N2, H10N7, H11N6, H12N5, H13N6, H14N5, H15N8 and H5N1.

In a preferred mode, the monoclonal antibody according to the present invention does not substantially undergo antigen-antibody reaction with the pathogens of other infectious diseases of which symptoms are at least partly similar to those of influenza. Thus, the preferred monoclonal antibody according to the present invention does not substantially undergo antigen-antibody reaction with, for example, adenovirus (types 1 to 7), Coxsackie virus (types A16 and B1 to B6), herpes simplex virus 1, echovirus (types 3, 4, 7, 22 and 30), enterovirus (type 71), mumps virus, poliovirus (types 1 to 3), RS virus (subgroups A and B), parainfluenza virus (types 1 to 3), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, myxomycete, *Staphylococcus aureus*, corynebacteria, *Corynebacterium diphtheriae*, *Candida albicans*, *Streptococcus pyogenes*, *Streptococcus sp.* (Groups B, C, G and F), *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Listeria monocytogenes*,

Mycoplasma pneumoniae, *Chlamydia trachomatis* and *Chlamydia pneumoniae*.

As is well-known, by decomposing an antibody with papain or pepsin, antibody fragments having binding ability with the corresponding antigen, such as Fab fragment and $F(ab')_2$ fragment (referred to as "antigen-binding fragment" in the present description) are obtained. In the present invention, the antigen-binding fragments of the monoclonal antibody according to the present invention may also be used in the same manner as the monoclonal antibody according to the present invention, and are within the scope of the present invention.

The monoclonal antibody according to the present invention may be prepared by the conventional hybridoma method using the nucleoprotein of influenza A virus as the immunogen. As the nucleoprotein of influenza A virus used as the immunogen, any of the virus culture medium, influenza HA vaccine, HA antigen for HI and recombinant antigen may be employed as long as the nucleoprotein exists abundantly and it can serve as an immunogen. To inhibit the actions of highly immunogenic HA and NA contained in the antigen, the nucleoprotein purified by ultracentrifugation (see, for example, J. Biochem.; 102:1241-1249,1987) or an antigen after treatment with a protease (see, for example, J. Immunol. Methods; 180:107-116, 1995) may be employed.

The monoclonal antibody may be produced by the hybridoma obtained by immunizing an animal with an antigen containing the above-described nucleoprotein of influenza A virus, and fusing a monoclonal antibody-producing cell of the animal with a tumor cell.

The above-described hybridoma may be obtained by the following method: That is, the nucleoprotein of influenza A virus obtained as described above, which is to be used as the antigen, is intraperitoneally or intravenously administered to an animal such as mouse dividedly in several times at 2- to 3-week intervals, together with Freund's complete adjuvant. Then antibody-producing cells originated from

spleen or the like are fused with tumor cells which can grow *in vitro*, such as myeloma cell line.

The fusion may be attained by using polyethylene glycol in accordance with the conventional method by Kohler and Milstein (Nature, Vol.256, 495, 1975) or by using Sendai virus or the like.

The selection of hybridomas which produce an antibody that recognizes the nucleoprotein of influenza A virus, from the fused cells may be carried out as follows: That is, from the fused cells, the cells which are alive in HAT medium and/or HT medium may be selected as hybridomas by limiting dilution method. Then the hybridomas producing the monoclonal antibody which specifically recognizes the nucleoprotein of influenza A virus may be selected by EIA or the like in which the culture medium of the hybridoma is reacted with highly purified nucleoprotein of influenza A virus immobilized on an assay plate, and then further reacted with anti-mouse immunoglobulin (Ig) or the like.

In Examples described below, a plurality of preferred monoclonal antibodies according to the present invention were obtained, and three of them were named hybridoma FVA2-3, hybridoma FVA2-6 and hybridoma FVA2-11, respectively. These hybridomas have been deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan, as international deposition in accordance with the Budapest Treaty. That is, hybridoma FVA2-3 has been deposited under identification reference of "FVA2-3" under accession No. FERM BP-10066 (date of deposit: July 18, 2003; converted, on July 14, 2004, from domestic deposition under accession No. FERM P-19437); hybridoma FVA2-6 has been deposited under identification reference of "FVA2-6" under accession No. FERM BP-10067 (date of deposit: July 18, 2003; converted, on July 14, 2004, from domestic deposition under accession No. FERM P-19438); and hybridoma FVA2-11

has been deposited under identification reference of "FVA2-11" under accession No. FERM BP-10068 (date of deposit: July 18, 2003; converted, on July 14, 2004, from domestic deposition under accession No. FERM P-19439).

Each of the above-described hybridomas may be recovered by culturing the hybridoma in a medium ordinarily used in cell cultures, and the monoclonal antibody may be recovered from the culture supernatant. Alternatively, the monoclonal antibody may be recovered by administering the hybridoma to an animal of the species from which the hybridoma derived to accumulate ascites therein, and by recovering the monoclonal antibody from the ascites.

For recovering the monoclonal antibody, usual purification methods may be employed. Examples of the purification methods include gel permeation chromatography, ion-exchange chromatography, affinity chromatography using protein A, and the like.

It was confirmed that the monoclonal antibodies prepared by the above-described method reacted with nucleoprotein of influenza A virus, and reacted with various subtypes of influenza A virus. They reacted with 38 stock strains (see Table 3 below) of influenza A virus from human, horse, duck, turkey, seal, chicken, gull, mallard and the like, and the stock strains were able to be detected by using monoclonal antibodies according to the present invention. On the other hand, cross-reactivities with the microorganisms, for example, adenovirus (types 1 to 7), Coxsackie virus (types A16 and B1 to B6), herpes simplex virus 1, echovirus (types 3, 4, 7, 22 and 30), enterovirus (type 71), mumps virus, poliovirus (types 1 to 3), RS virus (subgroups A and B), parainfluenza virus (types 1 to 3), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, myxomycete, *Staphylococcus aureus*, corynebacteria, *Corynebacterium diphtheriae*, *Candida albicans*, *Streptococcus pyogenes*, *Streptococcus sp.* (Groups B, C, G and F), *Streptococcus pneumoniae*, *Hemophilus*

influenzae, *Listeria monocytogenes*, *Mycoplasma pneumoniae*, *Chlamydia trachomatis* and *Chlamydia pneumoniae*, were checked, but no reaction was observed.

The monoclonal antibody according to the present invention may be used for immunoassays for the detection or quantification of influenza A virus. The immunoassays *per se* are well-known and any of the well-known immunoassays may be employed. That is, classifying the known immunoassays according to the reaction type, known immunoassays include sandwich immunoassays, competition immunoassays, agglutination immunoassays, Western blot and the like. Classifying the known immunoassays according to the label employed, known immunoassays include fluorescence immunoassays, enzyme immunoassays, radio immunoassays, biotin immunoassays and the like. Any of these immunoassays may be employed. Further, diagnosis may be attained by immunohistostaining. In cases where a labeled antibody is used in the immunoassay, the methods *per se* for labeling an antibody are well-known, and any of the well-known methods may be employed. It is known that by decomposing an antibody with papain or pepsin, an antibody fragment such as Fab fragment or $F(ab')_2$ fragment having the binding ability with the corresponding antigen (such a fragment is called "antigen-binding fragment" in the present specification) is obtained. The antigen-binding fragments of the antibody of the present invention may also be used in the same manner as the antibody of the present invention.

These immunoassays *per se* are well-known in the art, and so it is not necessary to explain these immunoassays in the present specification. Briefly, in sandwich immunoassays, for example, the antibody of the present invention or an antigen-binding fragment thereof is immobilized on a solid phase as a first antibody. The first antibody is then reacted with a sample, and after washing the solid phase, the resultant is then reacted with a second antibody which reacts with the enzyme of

the present invention by antigen-antibody reaction. After washing the solid phase, the second antibody bound to the solid phase is measured. By labeling the second antibody with an enzyme, fluorescent substance, radioactive substance, biotin or the like, measurement of the second antibody bound to the solid phase may be attained by measuring the label. The above-mentioned measurement is conducted for a plurality of standard samples each containing a known concentration of the enzyme, and the relationship between the concentrations of the enzyme in the standard samples and the measured amounts of the label is plotted to prepare a calibration curve. The enzyme in a test sample may be quantified by applying the measured amount to the calibration curve. It should be noted that the above-mentioned first antibody and the above-mentioned second antibody may be exchanged. In agglutination immunoassays, the antibody according to the present invention or an antigen-binding fragment thereof is immobilized on particles such as latex particles, and the particles are reacted with a sample, followed by measurement of the absorbance. The above-mentioned measurement is conducted for a plurality of standard samples each containing a known concentration of the enzyme, and the relationship between the concentrations of the enzyme in the standard samples and the measured absorbance is plotted to prepare a calibration curve. The enzyme in a test sample may be determined by applying the measured absorbance to the calibration curve.

The present invention also provides an immunoassay device using the above-described monoclonal antibody according to the present invention, by which influenza A virus may be detected simply in a manner distinguishing from influenza B virus.

The immunoassay device comprises a labeled reagent zone having a labeled mobile anti-influenza A virus antibody, a sample-spotting zone, a developer-supply zone, a developer-absorption zone and an influenza A virus-detection zone in which

an anti-influenza A virus antibody is immobilized on a matrix, which zones are provided in the matrix, wherein at least one of the labeled anti-influenza A virus antibody and the anti-influenza A virus antibody immobilized on the detection zone is the monoclonal antibody according to the present invention. The sample spotted on the sample-spotting zone undergoes antigen-antibody reaction with the labeled antibody contained in the labeled reagent zone, thereby a labeled antigen-antibody complex is formed. This antigen-antibody complex is flown by the developer supplied from the developer-supply zone to reach the influenza A virus-detection zone. Here, the anti-influenza A virus antibody immobilized on the matrix and the labeled antigen-antibody complex undergo antigen-antibody reaction so that the labeled antigen-antibody complex is immobilized on the matrix. Whether the antigen-antibody complex was immobilized on the influenza A virus-detection zone or not is judged by whether the label exists or not. The developer passed through the influenza A virus-detection zone is absorbed by the developer-absorption zone. The sample-spotting zone and the labeled reagent zone may be the same zone (in this case, the sample is spotted on the labeled reagent zone). In the immunoassay device according to the present invention, at least one of the labeled antibody and the antibody immobilized on the influenza A virus-detection zone is the monoclonal antibody according to the present invention. One of the antibodies may also be a polyclonal antibody. Since a plurality of nucleoprotein molecules of influenza A virus are usually associated or attached each other, influenza A virus may be detected even if the labeled antibody and the antibody immobilized on the influenza A virus-detection zone are the same monoclonal antibody.

The constituents of the immunoassay device according to the present invention will now be described separately.

Matrix

The belt-like matrix in the immunoassay device according to the present

invention is made of a water-absorptive material in which liquid can be transported by capillary action. Examples of the water-absorptive material include cellulose and derivatives thereof such as cellulose and nitrocellulose, and filter papers made of glass fibers alone or containing glass fibers, membranes and porous materials. Although the size of the matrix is not restricted, those in the form of strip having a width of about 3 mm to 10 mm, and a length of about 30 mm to 100 mm are preferred because they have good ease of handling. To prevent non-specific adsorption of the proteins originated from the sample to the matrix during the measurement, a part or the entire matrix may be blocked with an animal serum protein such as bovine serum albumin (BSA), casein, sucrose or the like.

Detection Zone

In the detection zone, an influenza A virus-detection section at which the anti-influenza A virus antibody is immobilized on the matrix may be provided. At least one of the anti-influenza A virus antibody immobilized on this detection section and the labeled anti-influenza A virus antibody described below is the anti-influenza A virus monoclonal antibody according to the present invention, and both of them are preferably the anti-influenza A virus monoclonal antibody according to the present invention. The anti-influenza A virus antibody on the detection section is preferably arranged on the matrix, in the form of a line perpendicular to the direction of the flow of the liquid (longitudinal direction of the matrix) for the measurement with high sensitivity. As the anti-influenza A virus polyclonal antibody, a polyclonal antibody may be appropriately selected from the commercially and easily available polyclonal antibodies.

The anti-influenza A virus antibody in the detection zone is the above-described antibody, and the monoclonal antibodies may be used individually or in combination. The anti-influenza A virus antibody may be IgG antibody or IgM antibody, or may be Fab, $F(ab')_2$ or the like which is an antigen-binding fragment of

these antibodies.

The anti-influenza A virus antibody immobilized on the detection section may be physically adsorbed directly on the detection zone in the matrix, or may be fixed by chemical bond such as covalent bond. Alternatively, the anti-influenza A virus antibody may be bound to a water-insoluble carrier and the carrier may be contained in the matrix. Examples of the insoluble carrier include the particles obtained by insolubilizing a mixture of gelatin, gum arabic and sodium hexametaphosphate (Japanese Patent Publication No. 63-29223), polystyrene latex particles, glass fibers and the like. The anti-influenza A virus monoclonal antibody may be bound to the insoluble carrier by the above-described chemical bond or by physical adsorption.

In the detection zone, an influenza B virus-detection section by an anti-influenza B virus antibody may be provided in addition to the influenza A virus-detection section by the anti-influenza A virus antibody. This influenza B virus-detection section may be arranged at either upstream or downstream of the influenza A virus-detection section in the direction of the flow of the developer, as long as it is arranged in the vicinity of the influenza A virus-detection section. The anti-influenza B virus antibody immobilized on the influenza B virus-detection section may be a polyclonal antibody or a monoclonal antibody, and may be immobilized on the matrix by chemical bond or by physical adsorption as in the case of the anti-influenza A virus antibody.

As mentioned above, it is preferred to provide an influenza B virus-detection section in addition to the influenza A virus-detection section in order to simultaneously measure the influenza A virus and influenza B virus. These detection sections are located downstream of the enzyme-labeled reagent zone, sample-spotting zone and developer-supply zone, and upstream of the developer-absorption zone, in the direction of the flow of the liquid in the matrix. The detection sections may be arranged on the matrix, in the form of a plurality of lines

each of which having a width of about 0.5 mm to 5 mm, which lines are closely arranged. In the case of a matrix having a width of about 5 mm, the detection sections may be formed by spotting the antibody and antigen usually in an amount of about 0.1 μg to 10 μg, respectively, and drying the matrix.

Labeled Reagent Zone

The labeled reagent zone may be formed by spotting a labeled anti-influenza A virus antibody movably in the zone provided on the matrix. This zone may be formed upstream of the detection zone in the direction of the flow of the developer from the developer-supply zone. This zone may be formed by spotting the enzyme-labeled reagent on the matrix, or by laminating a water-absorptive pad containing the enzyme-labeled reagent, or incorporating the enzyme-labeled reagent in a part or the entire region of the matrix which intimately contacts the pad. As the water-absorptive pad, the pad used for the sample-spotting zone hereinbelow described may be used.

At least one of the labeled anti-influenza A virus antibody and the antibody immobilized on the detection zone is the anti-influenza A virus monoclonal antibody according to the present invention, and both of them are preferably the anti-influenza A virus monoclonal antibody according to the present invention. As the labeled anti-influenza A virus antibody, fragments thereof may be employed as in the case of the above-described antibody in the detection zone.

The labeled anti-influenza A virus antibody may be prepared by binding the antibody with the label. Examples of the label include enzymes, colloidal metal particles, colored latex particles, luminescent substances, fluorescent substances and the like. As the enzyme, various enzymes used in enzyme immunoassays (EIA) may be employed. Examples of the enzyme include alkaline phosphatase, peroxidase, β-D-galactosidase and the like. Examples of the colloidal metal particles include colloidal gold particles, colloidal selenium particles and the like.

The label and the anti-influenza A virus antibody may be bound by the known methods using covalent bond or non-covalent bond. Examples of the binding method include glutaraldehyde method, periodate method, maleimide method, pyridyl disulfide method and methods using various cross-linking agents (see, for example, "PROTEIN, NUCLEIC ACID AND ENZYME", Extra Edition, vol.31, pp.37-45 (1985)). In the methods using a cross-linking agent, for example, *N*-succinimidyl-4-malcimide butyric acid (GMBS), *N*-succinimidyl-6-maleimide hexanoic acid, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid or the like may be used. In the methods using covalent bond, the functional groups existing in the antibody may be used. Alternatively, the labeled anti-influenza A virus antibody may be prepared by the above-described binding method after introducing a functional group such as thiol group, amino group, carboxyl group, hydroxyl group or the like. In the methods using non-covalent bond, the physical adsorption or the like may be used.

In cases where the influenza B virus is detected simultaneously with the detection of the influenza A virus as mentioned above, a labeled anti-influenza B virus antibody is added to the labeled reagent zone to prepare the device. The labeled anti-influenza A virus antibody and the labeled anti-influenza B virus antibody contained in the labeled reagent zone may be contained in one of the matrix and the water-absorptive pad, or may be contained in both of the matrix and the water-absorptive pad. In cases where the influenza A virus and the influenza B virus are simultaneously measured, since large amounts of the labeled reagents are used, it is advantageous for measurement with high sensitivity to incorporate the enzyme-labeled reagents individually or in combination in both of the matrix and the pad. Although the amount of the labeled anti-influenza A virus antibody and the labeled anti-influenza B virus antibody may be appropriately selected depending on the expected amount of the test substance in the sample, it is usually about 0.01 μg to

5 μg in terms of dry weight. When incorporating the labeled anti-influenza A virus antibody and the labeled anti-influenza B virus antibody in the enzyme-labeled reagent zone, they may be applied together with a stabilizer, solubilization-adjusting agent or the like.

Sample-spotting Zone

The sample-spotting zone may be formed in the matrix at a site downstream of the developer-supply zone and upstream of the detection zone in the direction of the flow of the developer, without incorporating a reagent or the like. The sample-spotting zone may also be formed at 1) a prescribed site downstream of the developer-supply zone and upstream of the enzyme labeled-reagent zone in the direction of the flow of the developer, 2) a prescribed site downstream of the labeled reagent zone and upstream of the detection zone in the direction of the flow of the developer, or 3) in a prescribed site on the labeled reagent zone. In the device in which the sample-spotting zone is formed in the enzyme labeled reagent zone, it is preferred to add the water-absorptive pad containing the enzyme label for carrying out the assay efficiently as mentioned above. With the device comprising the pad, since a large amount of sample fluid may be spotted, a minor component in the sample may be measured at a high detection sensitivity. The material constituting the water-absorptive pad is selected from the materials which scarcely adsorb the labeled reagent and the influenza virus. Examples of the materials include porous materials made of synthetic or natural macromolecular compounds such as polyvinyl alcohol (PVA), non-woven fabric, cellulose and the like, and these materials may be employed individually or in combination. Although the size, thickness, density and the like of the pad are not restricted, it is preferred to use a pad having longitudinal and lateral lengths of about 3 mm to 10 mm, and a thickness of about 0.5 mm to 4 mm for carrying out the assay efficiently.

Developer-supply Zone

The developer-supply zone is the zone formed at one end of the matrix in the longitudinal direction, to which the developer is supplied. The assay may be started by immersing this zone in the developer contained in a vessel in an amount sufficient to reach the developer-absorption zone. A liquid bath containing the developer may be attached to the developer-supply zone, and the assay may be started by breaking a cover of the liquid bath thereby bringing the developer into contact with the matrix. The developer may appropriately contain a surfactant, buffering agent, stabilizer, antibacterial agent or the like. In cases where an enzyme is used as the label, the substrate may be added to the developer in addition to the substrate reagent zone hereinbelow described. Examples of the buffer solution containing a buffer agent include acetate buffer, borate buffer, Tris-HCl buffer, diethanolamine buffer and the like. On the developer-supply zone, a developer pad may be mounted to stably and continuously supply the developer to the matrix. As the developer pad, a filter paper made of, for example, cellulose or cellulose derivative may be employed.

Developer-absorption Zone

The developer-absorption zone is provided in the matrix at the end other than the end at which the above-described developer-supply zone is provided. This zone is provided for absorbing the developer supplied to the matrix so as to fluently carry out the assay. The developer-absorption zone may be provided by elongating the matrix. A water-absorptive material may be arranged on the matrix to accelerate the development. As the water-absorptive material, filter papers having a high water holding capacity, made of natural macromolecular compounds, synthetic macromolecular compounds and the like, sponge or the like may be employed. Although the developer-absorption zone is made of an absorptive material in the form of a pad having a volume which can absorb the whole developer, a compact immunoassay device may be produced by laminating the absorptive material on or below the matrix.

Substrate Reagent Zone

In cases where an enzyme is used as the label contained in the labeled reagent zone, the substrate may be contained in the developer as mentioned above, or a substrate reagent zone may be provided in the matrix in the vicinity of the developer-supply zone. The substrate reagent zone may preferably be formed in the above-described developer pad formed on the developer-supply zone by incorporating the substrate in the developer pad.

As the substrate, various coloring substrate, fluorescent substrates, luminescent substrates and the like described below may be used depending on the enzyme in the labeled reagent.

(a) Coloring Substrates

For Peroxidase: 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) (ABTS), 3,3',5,5'-tetramethylbenzidine (TMB) and diaminobenzidine (DAB), in combination with hydrogen peroxide;

For Alkaline Phosphatase: 5-bromo-4-chloro-3-indolyl phosphate (BCIP)

(b) Fluorescent Substrates

For Alkaline Phosphatase: 4-methylumbelliferyl phosphate (4MUP)

For β-D-galactosidase: 4-methylumbelliferyl-β-D-galactoside (4MUG)

(c) Luminescent Substrates

For Alkaline Phosphatase: 3-(2'-spiroadamantan)-4-methoxy-4-(3"-phosphoryloxy)phenyl-1,2-dioxetane·2 sodium salt (AMPPD)

For β-D-galactosidase: 3-(2'-spiroadamantan)-4-methoxy-4-(3"-β-D-galactopyranosyl)phenyl-1,2-dioxetane (AMGPD)

For Peroxidase: luminol and isoluminol, in combination with hydrogen peroxide.

In cases where the substrate zone containing the substrate is provided, the substrate zone may usually be formed by applying an aqueous solution of the

substrate in the form of a line on the developer pad, and drying the solution. If desired, a signal-increasing agent, stabilizer, solubilization-adjusting agent or the like may be added. The site of the substrate zone is not restricted as long as it is within the developer pad mounted on the end of the matrix. The amount of the substrate added to the developer or the developer pad may be selected depending on the assay conditions, and may usually be about 5 μg to 500 μg per device.

Arrangement of Zones

A preferred embodiment of the immunoassay device according to the present invention is schematically shown in Figs. 6 to 8. In Fig. 6, reference numeral 2 denotes a matrix, reference numeral 4 denotes a labeled reagent zone, reference numeral 8 denotes a sample-spotting zone, reference numeral 3 denotes a developer-supply zone, reference numeral 5 denotes a developer-absorption zone, reference numeral 6a denotes an influenza A virus-detection zone, reference numeral 7 denotes a substrate reagent zone, and reference numeral 9 denotes a sample. In this embodiment, the sample-spotting zone 8 and the labeled reagent zone 4 are the same. Reference numeral 6a denotes an influenza B virus-detection zone, reference numeral 10 denotes a developer-confirmation zone and reference numeral 11 denotes a developer bath (see Example 6 below).

Method for Using Immunoassay Device

With the immunoassay device according to the present invention, influenza A virus in various samples may be assayed. The assay may be carried out by first supplying a sample to the sample-spotting zone of the immunoassay device, and then supplying the developer to the developer pad, thereby developing the sample in the matrix. As a diluent of the sample, a buffer containing a surfactant may be used. The developer moves in the matrix by capillary action to reach the developer-absorption zone. By the developer-absorption zone, the components in the sample, which were not bound to the detection zone, the enzyme-labeled reagent and the like

are absorbed, and the development is completed. After a prescribed time (usually 10 minutes to 20 minutes), the detection zone is observed, and the label bound to the detection section by the influenza A virus in the sample is measured, thereby measuring the influenza A virus. The detection may be carried out by visual observation or by using a measuring device such as colorimeter, fluorophotometer, photon counter, photosensitive film or the like, depending on the substrate, or the substrate and the enzyme used. For the measurement, the method in which the coloring of the detection zone is visually observed, for example, is simple. By this method, by using a color chart corresponding to the concentration of the influenza A virus, a semiquantitative assay may be attained. Quantification may also be attained by digitizing the coloring of the detection zone by a colorimeter or the like.

The principle of the assay will now be described in more detail taking the preferred embodiment of the immunoassay device according to the present invention shown in Figs. 6 to 8 as an example. A sample 9 is spotted on the sample-spotting zone 8, and the developer in the developer bath 11 is supplied to the developer-supply zone 3. The developer moves in the matrix 2 in the direction shown by the lateral open arrow by capillary action. The substrate contained in the substrate reagent zone 7 is dissolved in the developer and moves together with the developer. On the other hand, the sample 9 reacts with the labeled antibody in the labeled reagent zone 4, so that a labeled antigen-antibody complex is formed. When the developer reaches here, the labeled antigen-antibody complex moves in the matrix 2 while reacting with the substrate in the developer. When the labeled antigen-antibody complex reaches the influenza A virus-detection zone 6a, it reacts with the anti-influenza A virus antibody immobilized on the influenza A virus-detection zone 6a, thereby being immobilized on the influenza A virus-detection zone 6a. In cases where influenza A virus is contained in the sample, color is generated by the reaction between the label enzyme and the substrate. On the other hand, in cases where

influenza A virus is not contained in the sample, the labeled antibody cannot undergo antigen-antibody reaction and so is not immobilized on the influenza A virus-detection zone 6a, so that coloring does not occur. Therefore, whether the sample contains influenza A virus or not can be known based on whether the influenza A virus-detection zone 6a is colored or not. In the developer-confirmation zone 10, an enzyme which makes color by reaction with a reagent in the developer is immobilzed. Therefore, if the developer-confirmation zone 10 is colored, it indicates that the developer has reached there. Alternatively, an antibody to the enzyme label may be immobilized on the developer-confirmation zone 10. With this device, the labeled reagent which reached the developer-confirmation zone 10 is trapped by this antibody, and the trapped label and the substrate in the developer react so as to make color when the developer reached (in Examples below, anti-alkaline phosphatase antibody is immobilized). By incorporating a labeled anti-influenza B virus antibody in the labeled reagent zone 4, and by providing an influenza B virus-detection zone 6b, the detection of influenza B virus may also be carried out simultaneously, so that whether the influenza virus is A type or B type may be determined by a single assay operation.

The matrix may be laminated and fixed on a support made of a plastic, metal, paper or the like. By fixing the matrix in a case made of a plastic or the like, providing a bath containing the developer at the developer-supply zone, and covering the matrix with a case having through holes at the sites corresponding to the above-described zones, a device having a good ease of handling may be constituted. The sample to be assayed by the immunoassay device according to the present invention is a sample collected from human, animal or the like, which is thought to contain influenza virus. Examples of the sample include extracts of body fluids such as nasal swab, nasal aspirate, pharyngeal swab and the like.

The present invention will now be described in more detail by way of

Reference Examples and Examples, but the present invention is not limited to the Examples.

Examples

Example 1 Preparation of Anti-influenza A Virus Monoclonal Antibody

Influenza HA vaccine (produced by KAKETSUKEN: A/New Caledonia/20/99(H1N1)(IVR-116) strain, A/Panama/2007/99(H3N2)NIB-41) strain) containing the influenza nucleoprotein antigen was used as the immunogen. After completely mixing the immunogen with an equal amount of Freund's complete adjuvant (produced by IATRON), the immunogen was administered to a Balb/c mouse 4 times at 2-week intervals. Three days before the cell fusion, the immunogen was injected to the abdominal cavity of the mouse, and the mouse spleen cells and myeloma cells (P3U1) were fused using PEG. HAT selection medium was used as the culture medium. About 2 weeks later, the culture supernatant was collected and subjected to screening. In the primary screening, Enzyme Linked Immunosorbent Assay (ELISA) was employed, which used a solid phase on which influenza HA vaccine containing influenza nucleoprotein antigen and influenza A virus recombinant nucleoprotein antigens (r-NP/H1N1 originated from A/New Caledonia/20/99), r-NP/H3N2 originated from A/Kitakyushu/159/93; DDBJ/GenBank database) were immobilized. More particularly, the influenza HA vaccine was 300-fold diluted with 0.1 M carbonate buffer, pH9.6, and the type A recombinant nucleoprotein antigen was diluted to a concentration of 1 μg/ml with the same buffer. The respective obtained solutions were added to the wells of a microplate module (produced by NUNC) in an amount of 100 μl/well, and the microplate was incubated at 4°C overnight to immobilize the antigens. After washing each well with PBS containing 0.1% Tween 20 (trademark) (PBS-Tween), 300 μl of 1% bovine serum albumin (BSA) in PBS was added and the microplate was incubated at 4°C overnight, thereby carrying out blocking. After removing the

blocking solution, 100 µl of culture supernatant was added, and the resulting mixture was allowed to react at 37°C for 1 hour. After sufficiently washing each well with PBS-Tween, 100 µl of enzyme-labeled anti-mouse Igs antibody (produced by DAKO) 2000-fold diluted with 0.05 M phosphate buffer, pH7.5, containing 0.2% BSA, 0.2% Emulgen 985, 1% sucrose, 1% KCl (medium for reaction) was added to each well, and the mixture was allowed to react at 37°C for 1 hour. After the reaction, each well was sufficiently washed with PBS-Tween, and 100 µl of 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) (ABTS) was added to each well. After allowing each reaction mixture to react at room temperature for 30 minutes, 100 µl of a stop solution was added to each well, and the coloring level was measured at a primary wavelength of 415 nm and a secondary wavelength of 490 nm.

The culture supernatants judged as positive were subjected to secondary screening by ELISA using influenza B recombinant nucleoprotein (B/Yamanashi /166/98(r-NP/B)DDBJ/GenBank database), and 3 hybridoma strains (hybridoma FVA2-3, hybridoma FVA2-6 and hybridoma FVA2-11) each of which produces an antibody that reacts with influenza A virus nucleoprotein antigen and does not react with influenza B nucleoprotein antigen were finally obtained. These hybridomas have been deposited with International Patent Organism Depositary as described above. The subclass of all of the monoclonal antibodies obtained from these 3 hybridomas was IgG1. The reactivities of the hybridomas are shown in Table 1.

Table 1 Reactivities of Monoclonal Antibodies

	Antigen			
Monoclonal Antibody	r-NP/H1N1	r-NP/H3N2	Vaccine	r-NP/B
FVA2-3	+++	+++	2.206	0.018
FVA2-6	+++	+++	2.183	0.017
FVA2-11	+++	+++	2.223	0.018

+++, range over

Example 2 Reactivities of Monoclonal Antibodies in Western Blotting

The reactivities of the monoclonal antibodies were confirmed by Western blotting using the recombinant antigen, influenza HA vaccine (containing nucleoprotein antigen), and a virus solution. Each of the samples was dissolved in 0.01M Tris-HCl (pH8.0) containing 0.001M EDTA, 1%SDS, 5% 2ME (2-mercaptoethanol), 10% glycerol and 0.005% BPB (Bromophenol Blue), and each of the obtained solutions was heated to 100°C, followed by subjecting the resultant to electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by a conventional method using e-PAGEL, gel concentration 10% (produced by ATTO). After the electrophoresis, the bands were transferred to a PVDF membrane (produced by ATTO), and blocking was performed with Block Ace (produced by DAINIPPON PHARMACEUTICAL) at 4°C overnight. After removing the blocking solution and after washing the membrane with PBS-Tween, each monoclonal antibody solution adjusted to a concentration of 10 μg/ml was added, and the resultant was allowed to react at room temperature for 45 minutes. After sufficiently washing the membrane with PBS-Tween, an enzyme-labeled anti-mouse IgG antibody (produced by CAPPEL) 4000-fold diluted with the medium for reaction was added, and the resultant was allowed to react at room temperature for 45 minutes. After sufficiently washing the membrane with PBS-Tween, the membrane was exposed to an X-ray film (produced by KODAK) for 1 to 15 minutes using ECL Western Blotting Detection System (produced by AMERSHAM BIOSCIENCES) to detect signals.

Monoclonal antibodies FVA2-3, FVA2-6 and FVA2-11 gave bands at a site of about 55 kD to 70 kD to all of the antigens. The results are shown in Fig. 1.

Example 3 Measurement of Antigen by ELISA Using Monoclonal Antibody

A solution of each of the 3 types of purified monoclonal antibodies in 0.1 M phosphate buffer, pH7.5, having a concentration of 2 μg/ml was added to the wells of

a microplate module (produced by NUNC) in an amount of 100 µl per well, and the microplate was incubated at 4°C overnight to immobilize the antibody. Then each well was washed with PBS containing 0.1% Tween 20 (trademark) (PBS-Tween), and 300 µl of 1% bovine serum albumin (BSA) in PBS was added thereto, and the blocking was carried out at 4°C overnight. Influenza A virus solution dissolved in the medium for reaction was added to the wells in an amount of 100 µl per well, and the resultant was allowed to react at 37°C for 1 hour. After sufficiently washing each well with PBS-Tween, each of the monoclonal antibodies labeled with alkaline phosphatase dissolved in the medium for reaction was added to the wells in an amount of 100 µl/well, and the resultant was allowed to react at 37°C for 1 hour. After the reaction, each well was sufficiently washed with PBS-Tween, and 100 µl of 4-nitrophenyl phosphate (4-NPP) was added to each well. After reaction at room temperature for 30 minutes, stop solution was added to the wells in an amount of 100 µl/well. The coloring level was measured at a primary wavelength of 415 nm and a secondary wavelength of 490 nm. Those wherein the coloring level was not less than 2.1 times that of the buffer blank were judged as positive, and the reactivity was expressed in terms of the final dilution factor at which the sample was last judged as positive.

All of the 9 combinations of the obtained three types of monoclonal antibodies showed 2 to 8 times higher reactivity than the commercially available anti-influenza A virus monoclonal antibodies. The results are shown in Table 2.

Table 2 ELISA for Measuring Antigens

Monoclonal Antibody		Antigen			
Enzyme-labeled Antibody	Immobilized Antibody	A / Sydney /5/97	A / Beijin /352/89	A / New Caledonia /20/99	A / Panama /2007/99
FVA2-3	FVA2-3	x640	x80	x320	x640
	FVA2-6	x640	x160	x320	x640
	FVA2-11	x640	x160	x320	x640
FVA2-6	FVA2-3	x640	x80	x320	x640
	FVA2-6	x640	x160	x320	x640
	FVA2-11	x640	x80	x320	x640
FVA2-11	FVA2-3	x640	x80	x320	x640
	FVA2-6	x640	x160	x320	x640
	FVA2-11	x640	x160	x320	x640
commercial antibody*	commercial antibody*	x320	x40	x80	x80

* commercially available monoclonal antibody

Example 4 Determination of Recognition Site by Anti-influenza A Virus Monoclonal Antibody

4-1 Construction of Plasmids

To determine the recognition site by monoclonal antibody FVA2-11, three fragments encoding 1st to 159th amino acids (A fragment), 162nd to 327th amino acids (B fragment) and 327th to 498th amino acids, respectively, of the gene of influenza H1N1 strain (New Caledonia/20/99) encoding 498 amino acids were amplified by PCR. The primers preliminarily had EcoRI and BamHI sites. The amplified fragments were purified by PCR Purification Kit commercially available from QIAGEN, and each fragment was inserted to the EcoRI-BamHI site of expression plasmid pWG6A obtained by inserting GST in the NdeI-EcoRI site of the expression plasmid pW6A shown in Fig. 2, to prepare plasmids pWGInf.H1N1-A, pWGInf.H1N1-B and pWGInf.H1N1-C. *E. coli* BL21(DE3) (obtained from Brookhaven National Laboratory) was transformed with each plasmid to obtain ampicillin-resistant *E. coli* transformants BL21(DE3)pWGInf.H1N1-A, BL21(DE3)pWGInf.H1N1-B and BL21(DE3)pWGInf.H1N1-C.

4-2 Expression of Recombinant Proteins (GST+H1N1-A, GST+H1N1-B and GST+H1N1-C)

Each of the transformants prepared in 4-1 was cultured in 2 ml of LB medium containing 50 μg/ml of ampicillin at 37°C. After adjusting the OD at 600 nm to 0.6 to 0.8 in preliminary culture, 0.4 mM IPTG was added to induce expression, and the transformants were cultured for another 3 hours. The bacterial culture medium in an amount of 1.5 ml was centrifuged at 5000 rpm for 2 minutes to collect the cells, and the cells were suspended in 100 μl of a buffer (10 mM Tris-HCl, pH8.0, containing 0.1 M sodium chloride and 1 mM EDTA), followed by sonication for 15 minutes to completely disrupt the cells. The resultant was used as the bacterial sample.

To 8 μl of the bacterial sample, 4 μl of SDS polyacrylamide buffer having 3 times concentration (0.15 M Tris-HCl, pH6.8, 6% SDS, 24% glycerol, 6mM EDTA, 2% 2-mercaptoethanol, 0.03% bromphenol blue) was added, and the mixture was well stirred, followed by subjecting the mixture to SDS-polyacrylamide gel electrophoresis. The bands were transferred to a nitrocellulose filter by Western blot, and, after blocking with 1% BSA, monoclonal antibody FVA2-11 1000-fold diluted with phosphate buffer (10 mM phosphate, pH7.4, 0.15M sodium chloride) was allowed to react. Then anti-mouse immunoglobulin rabbit polyclonal antibody (produced by DAKO) labeled with peroxidase was allowed to react, and, after washing, 10 ml of substrate coloring solution (0.01% aqueous hydrogen peroxide solution, 0.6 mg/ml 4-chloro-1-naphthol) was added to make color. As a result, it was proved that monoclonal antibody FVA2-11 reacted with GST+H1N1-A alone.

4-3 Construction of Plasmids

Fragments obtained by further subdividing the A fragment, that is, A1 (1st to 90 amino acids), A2 (85th to 159th amino acids), A3 (44th to 130th amino acids), A4 (59th to 103rd amino acids), A5 (44th to 103rd amino acids), A6 (59th to 130 amino acids) and A7 (1st to 130 amino acids) (Fig. 3), were amplified by PCR. Each of the amplified fragments was purified by PCR Purification Kit commercially available

from QIAGEN, and was inserted into the EcoRI-BamHI site of the above-described expression plasmid pWG6A to prepare plasmids pWGInf.H1N1-A1, pWGInf.H1N1-A2, pWGInf.H1N1-A3, pWGInf.H1N1-A4, pWGInf.H1N1-A5 and pWGInf.H1N1-A6. A7 fragment was inserted into the EcoRI-BamHI site of pW6A shown in Fig. 2 to prepare a plasmid pWInf.H1N1-A7. *E. coli* BL21(DE3) (obtained from Brookhaven National Laboratory) was transformed with each plasmid to obtain ampicillin-resistant *E. coli* transformants BL21(DE3)pWGInf.H1N1-A1, Bl21(DE3)pWGInf.H1N1-A2, BL21(DE3)pWGInf.H1N1-A3, BL21(DE3)pWGInf.H1N1-A4, BL21(DE3)pWGInf.H1N1-A5, BL21(DE3)pWGInf.H1N1-A6 and BL21(DE3)pWInf.H1N1-A7.

4-4 Expression of Recombinant Proteins (GST+H1N1-A1, GST+H1N1-A2, GST+H1N1-A3, GST+H1N1-A4, GST+H1N1-A5, GST+H1N1-A6 and H1N1-A7)

Each of the transformants prepared in 4-3 was cultured in 2 ml of LB medium containing 50 μg/ml of ampicillin at 37°C. After adjusting the OD at 600 nm to 0.6 to 0.8 in preliminary culture, 0.4 mM IPTG was added to induce expression, and the transformants were cultured for another 3 hours. The bacterial culture medium in an amount of 1.5 ml was centrifuged at 5000 rpm for 2 minutes to collect the cells, and the cells were suspended in 100 μl of a buffer (10 mM Tris-HCl, pH8.0, containing 0.1 M sodium chloride and 1 mM EDTA), followed by sonication for 15 minutes to completely disrupt the cells. The resultant was used as the bacterial sample.

To 8 μl of the bacterial sample, 4 μl of SDS polyacrylamide buffer having 3 times concentration (0.15 M Tris-HCl, pH6.8, 6% SDS, 24% glycerol, 6mM EDTA, 2% 2-mercaptoethanol, 0.03% bromphenol blue, molecular weight markers (117.6, 113.9, 81.2, 60.7, 47.4, 36.1, 25.3, 19.0, 14.7, 6.1KD)) was added, and the mixture was well stirred, followed by subjecting the mixture to SDS-polyacrylamide gel electrophoresis. The bands were transferred to a nitrocellulose filter by Western

blot, and, after blocking with 1% BSA, monoclonal antibody FVA2-11 1000-fold diluted with phosphate buffer (10 mM phosphate, pH7.4, 0.15M sodium chloride) was allowed to react. Then anti-mouse immunoglobulin rabbit polyclonal antibody (produced by DAKO) labeled with peroxidase was allowed to react, and, after washing, 10 ml of substrate coloring solution (0.01% aqueous hydrogen peroxide, 0.6 mg/ml 4-chloro-1-naphthol) was added to make color. Each fragment is shown in Fig. 3. The CBB staining pattern after electrophoresis and the results of the Western blot are shown in Fig. 4. From these results, it can be seen that the recognition site of the anti-influenza A virus monoclonal antibody (FVA2-11) is the region of the 59th to 130th amino acids of influenza H1N1. From the amino acid sequences of the nucleoproteins of the influenza A subtypes shown in Fig. 5, the common sequence in the reaction site of the 59th to 130th amino acids, which has a hydrophilic structure, that is, for example, the amino acid sequences of 90th to 95th amino acids, 111st to 115th amino acids, 120th to 123rd amino acids or the like is expected to be the epitope.

Reference Example 1 Preparation of Anti-influenza A Virus Monoclonal Antibody Labeled with Alkaline Phosphatase

The anti-influenza A virus monoclonal antibody (FVA2-11) was treated with pepsin to cut off the Fc region to obtain $F(ab')_2$, and the disulfide bond was cleaved by 2ME (2-mercaptoethanol, produced by NACALAI TESQUE) to obtain $F(ab')_2$ of which free thiol group is exposed. Then alkaline phosphatase into which a maleimide group was introduced was coupled with the $F(ab')_2$, and the resultant was purified by gel permeation chromatography to obtain purified anti-influenza A virus antibody labeled with alkaline phosphatase.

Reference Example 2 Preparation of Anti-influenza B Virus Monoclonal Antibodies

Influenza HA vaccine (B/Yamanashi/166/98 strain) containing the influenza

nucleoprotein antigen, or influenza A recombinant nucleoprotein antigen (r-NP/H1N1 originated from A/New Caledonia/20/99 strain or r-NP/H3N2 originated from A/Kitakyushu/159/93) was used as the immunogen.

After completely mixing the immunogen with an equal amount of Freund's complete adjuvant (produced by IATRON), the immunogen was administered to a Balb/c mouse 4 times at 2-week intervals. Three days before the cell fusion, the immunogen was injected to the abdominal cavity of the mouse, and the mouse spleen cells and myeloma cells (P3U1) were fused using PEG. HAT selection medium was used as the culture medium. About 2 weeks later, the culture supernatant was collected and subjected to screening. In the primary screening, Enzyme Linked Immunosorbent Assay (ELISA) was employed, which used a solid phase on which influenza HA vaccine and recombinant nucleoprotein antigen of influenza B virus (r-NP/B) were immobilized. More particularly, the influenza HA vaccine was 300-fold diluted with 0.1 M carbonate buffer, pH9.6, and the type B recombinant nucleoprotein antigen was diluted to a concentration of 1 μg/ml with the same buffer. The respective obtained solutions were added to the wells of a microplate module (produced by NUNC) in an amount of 100 μl/well, and the microplate was incubated at 4°C overnight to immobilize the antigens. After washing each well with PBS containing 0.1% Tween 20 (trademark) (PBS-Tween), 300 μl of 1% bovine serum albumin (BSA) in PBS was added and the microplate was incubated at 4°C overnight, thereby carrying out blocking. After removing the blocking solution, 100 μl of culture supernatant was added, and the resulting mixture was allowed to react at 37°C for 1 hour. After sufficiently washing each well with PBS-Tween, 100 μl of enzyme-labeled anti-mouse Igs antibody (produced by DAKO) 2000-fold diluted with 0.05 M phosphate buffer, pH7.5, containing 0.2% BSA, 0.2% Emulgen 985, 1% sucrose and 1% KCl (medium for reaction) was added to each well, and the mixture was allowed to react at 37°C for 1 hour. After the reaction, each well was

sufficiently washed with PBS-Tween, and 100 μl of 2,2'-azino-bis(3-ethylbenzothiazolin-6-sulfonate) (ABTS) was added to each well. After allowing each reaction mixture to react at room temperature for 30 minutes, 100 μl of a stop solution was added to each well, and the coloring level was measured at a primary wavelength of 415 nm and a secondary wavelength of 490 nm.

The culture supernatants judged as positive were subjected to secondary screening by ELISA using influenza A recombinant nucleoproteins (r-NP/H1N1 originated from A/New Caledonia/20/99) and r-NP/H3N2 originated from A/Kitakyushu/159/93), and 3 hybridoma strains (hybridoma FrB1-03, hybridoma FrB1-07 and hybridoma FVB2-16) each of which produces an antibody that reacts with influenza B virus nucleoprotein antigen and does not react with influenza A nucleoprotein antigen were finally obtained. These hybridomas have been deposited with International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology. Hybridoma FrB1-03 has been deposited under an accession No. FERM BP-10070, hybridoma FrB1-07 has been deposited under an accession No. FERM BP-10071, and hybridoma FVB2-16 has been deposited under an accession No. FERM BP-10069. The subclass of all of the monoclonal antibodies obtained from these 3 hybridomas was IgG1.

(Reactivities of Monoclonal Antibodies in Western Blotting)

The reactivities of the monoclonal antibodies were confirmed by Western blotting using the recombinant antigen and influenza HA vaccine (containing nucleoprotein antigen). SDS-PAGE was carried out by a conventional method using e-PAGEL, gel concentration 10% (produced by ATTO). After the electrophoresis, the bands were transferred to a PVDF membrane (produced by ATTO), and blocking was performed with Block Ace (produced by DAINIPPON PHARMACEUTICAL) at 4°C overnight. After removing the blocking solution and after washing the membrane with PBS-Tween, each monoclonal antibody solution

adjusted to a concentration of 10 μg/ml was added, and the resultant was allowed to react at room temperature for 45 minutes. After sufficiently washing the membrane with PBS-Tween, an enzyme-labeled anti-mouse IgG antibody (produced by CAPPEL) 4000-fold diluted with the medium for reaction was added, and the resultant was allowed to react at room temperature for 45 minutes. After sufficiently washing the membrane with PBS-Tween, the membrane was exposed to an X-ray film (produced by KODAK) for 1 minute using ECL Western Blotting Detection System (produced by AMERSHAM BIOSCIENCES) to detect signals.

With monoclonal antibodies FrB1-03 and FrB1-07, a strong band was observed at about 60 to 75 kD.

Reference Example 3 Preparation of Anti-influenza B Virus Antibody Labeled with Alkaline Phosphatase

Using the anti-influenza B virus monoclonal antibody (FrB1-03) prepared in Reference Example 2, anti-influenza B virus monoclonal antibody labeled with alkaline phosphatase was obtained by repeating the same operations as in Reference Example 1 described above.

Example 5 Immunoassay Device for Simultaneous Assay of Influenza A Virus and Influenza B Virus

As shown in Fig. 6, on the sites 16 mm and 13.5 mm from the end, of the side of the developer-absorption zone 5, of the matrix 2 made of nitrocellulose membrane (produced by MILLIPORE), 0.7 μl of an aqueous solution of anti-influenza A virus antibody (FVA2-11) prepared in Example 2 and 0.7 μl of an aqueous solution of anti-influenza B virus antibody (FrB1-3) prepared in Reference Example 2 were spotted, respectively, and dried to form the detection zones 6a and 6b. At the site of 11 mm from the end, of the side of the developer-absorption zone 5, of the matrix 2, anti-alkaline phosphatase antibody (produced by DAKO) was spotted and dried to form the developer-confirmation zone 10. Then 5 μl of a mixed aqueous solution of the

alkaline phosphatase-labeled anti-influenza A virus antibody (5 μg/ml) prepared in Reference Example 1 and the alkaline phosphatase-labeled anti-influenza B virus antibody (5 μg/ml) prepared in Reference Example 3 was spotted on the matrix and dried to form a labeled reagent zone constituted by the enzyme label reagent pad 4.

The developer pad 3 was formed by spotting 100 μg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate in the form of a line having a width of 6.1 mm on a filter paper (produced by MILLIPORE) having a width of 5 mm and a length of 20 mm, and drying the substrate. The above-described matrix 2, developer pad 3, enzyme-labeled reagent pad 4 and the developer-absorption pad 5 (a filter paper (produced by MILLIPORE) having a width of 10 mm, length of 20 mm and a thickness of 1 mm) were fixed in a plastic case having the developer bath 11 to produce the immunoassay device 1 for simultaneous assay of influenza A virus and influenza B virus, shown in Figs. 7 and 8.

Reference Example 4 Immunoassay Device for Simultaneous Assay of Influenza A Virus and Influenza B Virus (conventional device)

The same immunoassay device 1 for simultaneous assay of influenza A virus and influenza B virus prepared in Example 3 was prepared as in Example 3 except that the detection zones 6a and 6b were formed by using a purchased anti-influenza A virus monoclonal antibody and a purchased anti-influenza B virus monoclonal antibody, respectively, to prepare an immunoassay device for simultaneous assay of influenza A virus and influenza B virus (ESPLINE Influenza A&B (produced by FUJIREBIO); conventional immunoassay device).

Example 6 Assay of Influenza A Virus and Influenza B Virus

On the sample-spotting zone 8 of the assay device 1 for simultaneous assay of influenza A virus and influenza B virus (the assay device of the present invention) prepared in Example 5, 30 μl of a sample of the subtype of influenza A virus shown in Table 3 (Tris buffer (pH8.0) containing a surfactant was used as the sample

diluent) was spotted, and then a pushing area 12 in a deformable member was pushed down to deform the same so as to immerse the developer pad 3 into the developer bath 11 with a projection 3 provided on the deformable member, thereby starting the assay. Fifteen minutes after the start of the assay, development of the developer was confirmed by the coloring of the developer-confirmation zone 10, and then the coloring of the detection zones 6a and 6b was visually observed. The results are shown in Table 3.

As a control, 30 μl each of the above-described sample shown in Table 3 was subjected to the assay using the conventional assay device prepared in Reference Example 4. The assay results are shown in Table 3.

Table 3 Results of Assay of Influenza A Virus Subtypes

Strain	Subtype	Conventional Assay Device		Assay Device of the Invention	
		Coloring 6a	Coloring 6b	Coloring 6a	Coloring 6b
A/Puerto Rico/8/34	H1 N1	+	−	+	−
A/Singapore/1/57	H2 N2	+	−	+	−
A/Aichi/2/68	H3 N2	+	−	+	−
A/equine/Miami/1/63	H3 N8	+	−	+	−
A/equine/Tokyo/2/71	H3 N8	+	−	+	−
A/equine/Kentucky/1/81	H3 N8	−	−	+	−
A/equine/Suffork/89	H3 N8	+	−	+	−
A/equine/Alaska/1/91	H3 N8	+	−	+	−
A/equine/Kentucky/1/91	H3 N8	−	−	+	−
A/equine/Rome/5/91	H3 N8	−	−	+	−
A/equine/Taby/91	H3 N8	−	−	+	−
A/equine/Lambourn/22778/92	H3 N8	−	−	+	−
A/equine/Hong Kong/92	H3 N8	−	−	+	−
A/equine/Ayesta/1/93	H3 N8	−	−	+	−
A/equine/La Plata/1/93	H3 N8	+	−	+	−
A/equine/Kentucky/1/94	H3 N8	−	−	+	−
A/equine/La Plata/1/95	H3 N8	+	−	+	−
A/equine/La Plata/1/96	H3 N8	+	−	+	−
A/duck/Ozech/56	H4 N6	−	−	+	−
A/duck/Pennsylvania/10128/83	H5 N2	+	−	+	−
A/turkey/Massachusetts/3740/65	H6 N2	+	−	+	−
A/seal/Massachusetts/1/80	H7 N7	+	−	+	−
A/equine/Prague/1/56	H7 N7	−	−	+	−
A/equine/Newmarket/1/77	H7 N7	−	−	+	−
A/turkey/Ontario/67	H8 N4	−	−	+	−
A/turkey/Wisconsin/66	H9 N2	−	−	+	−
A/chicken/Germany/N/49	H10 N7	−	−	+	−
A/duck/England/56	H11 N6	−	−	+	−
A/duck/Alberta/60/76	H12 N5	−	−	+	−
A/gull/Maryland/704/77	H13 N6	−	−	+	−
A/mallard/Astrakhan/263/82	H14 N5	+	−	+	−
A/duck/Australia/341/83	H15 N8	+	−	+	−
A/Hokkaido/11/02	H1 N1	+	−	+	−
A/Hokkaido/1/03 *	H3 N2	+	−	+	−
A/swine/Miyagi/5/03	H1 N2	+	−	+	−
A/HongKong/483/97	H5 N1	−	−	+	−
A/HongKong/156/97	H5 N1	−	−	+	−
A/HongKong/reverse genetics-911	H5 N1	+	−	+	−
Control		−	−	−	−

* Assay results of MDCK cultere supernatant because the viruses had not been habituated to chicken egg.

As seen from the results, all of the subtypes of influenza A virus which could not be detected by the conventional assay device were able to be detected by the assay device according to the present invention.

Example 7

Using the assay device 1 for simultaneous assay of influenza A virus and

influenza B virus (the assay device of the present invention) prepared in Example 5, cross-reactivities with adenovirus (types 1 to 7), Coxsackie virus (types A16 and B1 to B6), herpes simplex virus 1, echovirus (types 3, 4, 7, 22 and 30), enterovirus (type 71), mumps virus, poliovirus (types 1 to 3), RS virus (subgroups A and B), parainfluenza virus (types 1 to 3), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus epidermidis*, myxomycete, *Staphylococcus aureus*, corynebacteria, *Corynebacterium diphtheriae*, *Candida albicans*, *Streptococcus pyogenes*, *Streptococcus sp.* (Groups B, C, G and F), *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Listeria monocytogenes*, *Mycoplasma pneumoniae*, *Chlamydia trachomatis* and *Chlamydia pneumoniae*, were checked in the same manner as in Example 6. As a result, the device did not react with any of these viruses and bacteria.

CLAIMS

1. An anti-influenza A virus monoclonal antibody which undergoes antigen-antibody reaction with a nucleoprotein having a molecular weight of 55 kD to 70 kD of influenza A virus and which does not substantially undergo antigen-antibody reaction with influenza B virus, or an antigen-binding fragment thereof.

2. The monoclonal antibody or the antigen-binding fragment thereof according to claim 1, which reacts with an epitope existing in the region between 59th and 130th amino acids of the nucleoprotein of influenza A virus.

3. The monoclonal antibody or the antigen-binding fragment thereof according to claim 1 or 2, which undergoes antigen-antibody reaction with each of the subtypes of influenza A virus.

4. The monoclonal antibody or the antigen-binding fragment thereof according to claim 1, which monoclonal antibody is produced by hybridoma FVA2-3 deposited under an accession No. FERM BP-10066, hybridoma FVA2-6 deposited under an accession No. FERM BP-10067 or by hybridoma FVA2-11 deposited under an accession No. FERM BP-10068, which were deposited as international deposition in accordance with the Budapest Treaty.

5. An immunoassay device comprising a labeled reagent zone having a labeled mobile anti-influenza A virus antibody, a sample-spotting zone, a developer-supply zone, a developer-absorption zone and an influenza A virus-detection zone in which an anti-influenza A virus antibody is immobilized on a matrix, which zones are provided in said matrix, wherein at least one of said labeled anti-influenza A virus antibody and said anti-influenza A virus antibody immobilized on said detection zone is said monoclonal antibody according to any one of claims 1 to 4.

6. The immunoassay device according to claim 5, further comprising a labeled anti-influenza B virus antibody in said labeled reagent zone, and an influenza B virus-detection zone on which an anti-influenza B virus antibody is immobilized,

which influenza B virus-detection zone is located in the vicinity of said influenza A virus-detection zone on which said anti-influenza A virus antibody is immobilized.

7. The immunoassay device according to claim 5 or 6, wherein said label is an enzyme, and said device comprises a substrate for said enzyme, in said developer and/or in the vicinity of said developer-supply zone.