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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 November 2007 (01.11.2007)

PCT

(10) International Publication Number
WO 2007/122517 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/IB2007/002494

(22) International Filing Date: 26 April 2007 (26.04.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2541872 26 April 2006 (26.04.2006) CA

(71) Applicants *(for all designated States except US)*: **HKU-PASTEUR RESEARCH CENTRE LIMITED** [CN/CN]; Dexter HC Man Building, 8, Sassoon Road, Pokfulam, Hong Kong (CN). **INSTITUT PASTEUR DU CAMBODGE** [KH/KH]; 5, Boulevard Monivong, Phnom Pen (KH). **INSTITUT PASTEUR** [FR/FR]; 25-28, rue du Docteur Roux, F-75724 Paris Cédex 15 (FR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **NEFKENS, Isabelle** [BE/CN]; HKU-Pasteur Research Centre, H.C. Man Building, 8, Sassoon Road, Pokfulam, Hong Kong (CN). **ALTMEYER, Ralf** [DE/SG]; 19 Cairnhill Circle #17-06, The Light, Singapore 229 768 (SG). **GARCIA, Jean-Michel** [FR/CN]; HKU-Pasteur Research Centre, H.C. Man Building, 8, Sassoon Road, Pokfulam, Hong Kong (CN). **BUCHY, Philippe** [FR/FR]; Les Bastides du Golfe, 1295 Chemin des Eucalyptus (FR). **PEIRIS, Malik** [GB/CN]; 19th Floor, 29 Bagiuo Villa, 550 Victoria Road, Pokfulam, Hong Kong (CN).

(74) Agents: **DESAIX, Anne** et al.; Ernest Gutmann - Yves Plasseraud SAS, 3, rue Auber, F-75009 Paris (FR).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, 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, KR, 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, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, 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:
— *without international search report and to be republished upon receipt of that report*
— *the filing date of the international application is within two months from the date of expiration of the priority period*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/122517 A2

(54) Title: H5 PSEUDOTYPED VIRUSES AND USES THEREOF

(57) Abstract: The present invention relates to the field of Influenza in the presence of Influenza antibodies and, more particularly, it relates to the use of hemagglutinin pseudotyped viruses in methods for detecting the presence or absence of Influenza antibodies in a sample. The present invention also relates to the use of the hemagglutinin pseudotyped viruses in methods for the detection of modulators of influenza virus entry in a cell.

H5 PSEUDOTYPED VIRUSES AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to the field of Influenza in the presence of Influenza antibodies and, more particularly, the present invention relates to the use of hemagglutinin pseudotyped viruses in methods for detecting the presence or absence of Influenza antibodies in a sample. The present invention also relates to the use of the hemagglutinin pseudotyped viruses in methods for the detection of modulators of influenza virus entry in a cell.

BACKGROUND OF THE INVENTION

The global spread of highly pathogenic avian influenza (HPAI) A H5N1 viruses in poultry and its transmission to humans poses a pandemic threat. Since 2003 there have been over 270 human cases with over 160 deaths. Binding of influenza virus to cellular receptors is determined by the viral hemagglutinin. The HA1 subunit of hemagglutinin binds to terminal sialic acids of glycoproteins and glycolipids at the cell surface (Skehel and Wiley, 2000; Skehel and Wiley, 2002). Avian influenza viruses preferentially bind α2-3-linked sialic acids (SA) (Skehel et al., 1982; Skehel et al., 1983, Russell et al., 2006) while human influenza viruses preferentially recognize α2-6-linked SA. Subsequent virus entry and uncoating is dependent on low pH (Skehel and Wiley, 2000). During the final stage of the virus life cycle the HA binds to the SA receptor requiring the enzymatic activity of the neuraminidase for the release of the viruses from the cell surface (Dong et al. 1992).

Twelve percent of household contacts of confirmed H5N1 patients during the H5N1 outbreak in Hong Kong in 1997 had neutralizing antibodies against the H5N1 (Katz et al., 1999). However, seroepidemiologic studies during the recent H5N1 outbreaks indicate a low transmission rate of H5N1 virus to humans in spite of extensive exposure to infected poultry (Vong et al., 2006). At present, microneutralization tests confirmed by western-blot assays are the gold-standard for detection of anti-H5 specific antibodies in humans (Rowe et al., 1999). However H5N1 microneutralization tests require BSL-3 containment which precludes such studies in many affected countries. The conventional haemagglutination inhibition (HI) test is not suitable for serodiagnosis of avian H5N1 infections in humans (Rowe et al 1999). The modified HI test using horse erythrocytes (Stephenson et al., 2003) is currently under evaluation but is not a functional assay for inhibition of entry and is not amenable to high throughput testing.

There is thus a need to definite need for the development of new tools for the serodiagnosis of Influenza antibodies, and for the screening of modulators with respect of Influenza entry into cells.

SUMMARY OF THE INVENTION

An aspect of the invention is to provide new serodiagnostic tools and new inhibiting or promoting compounds in the field of Influenza.

Such an aspect is particularly achieved by providing a method for detecting the presence or absence of Influenza antibodies in a sample, comprising the steps of:

a) contacting the sample with an hemagglutinin pseudotyped virus under conditions sufficient to form an immune complex; and

b) detecting the presence or absence of the immune complex formed in a).

Said method is especially performed *in vitro* on a sample previously obtained from a subject to be diagnosed.

Another aspect of the invention concerns isolated and purified hemagglutinin pseudotyped virus, wherein said hemagglutinin is encoded by :

- a polynucleotide encoding an H1 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 2;
- a polynucleotide encoding an H3 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 3;
- a polynucleotide encoding an H7 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 4 or
- a polynucleotide encoding an H5 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO1 or 5.

Further aspects of the invention concern the use of an hemagglutinin pseudotyped virus as defined above in methods for the detection of a compound that either inhibits or promotes the entry of an Influenza virus in a cell.

Yet, another aspect of the invention provides a kit for the detection of the presence or absence of antibodies indicative of Influenza virus, comprising:

- an hemagglutinin pseudotyped virus as defined above;
- a reagent to detect an hemagglutinin-antibody immune complex;
- optionally a biological reference sample lacking antibodies that immunologically bind with said hemagglutinin; and
- optionally a comparison sample comprising antibodies which can specifically bind to said hemagglutinin;

wherein said hemagglutinin pseudotyped virus, reagent, biological reference sample, and comparison sample are present in an amount sufficient to perform said detection.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A: Production of lentiviral particles pseudotypes with a synthetic H5 envelope protein.

1. HEK293 T cells ("producer cells") are transfected with provirus expressing the luciferase or GFP reporter gene driven by the LTR promoter and a plasmid expressing the synthetic H5 gene. The cells will subsequently secrete pseudotyped viruses containing the proviral RNA and surrounded by the synthetic H5 gene. **2.** 12 h after transfection NA was added to the medium to release the particles that were bound to the sialic acids that are present on the cell surface of the producer cells. **3.** 24 h later, the supernatant containing the secreted pseudotyped viruses is harvested, filtered and incubated with the target cells. **4.**The synH5 pp will interact with their receptors and the HA2 will fuse with the plasma membrane. 5. After endocytosis, the nucleopcapsid is released into the cytoplasm. **6.** The nucleocapsid will then brake down and the viral mRNA is reverse transcribed into DNA. Finally, the viral DNA is transported into the nucleus where it will be integrated into the DNA of the host cell. There it will express the reporter gene which can be detected by a lucifease assay or FACS analysis for the luciferase or GFP gene respectively.

Figure 1B: Expression of H5 in the producer cells.

A western assay was performed on cell lysates from the HEK293T producer cells. The blot was then stained with a human serum against H5N1. Lane 1: HEK293T cells, lane 2: HEK293T cells transfected with pNL Luc E- R- and pCDNA-synH5.

Figure 2A: The yield of H5 pp in the supernatant is increased in the presence of sNA.

sNA was added to the HEK293T producer cell line after transfection. 24 h later, the supernatant was harvested, filtered and incubated with Huh7 cells. The yield is expressed in relative luciferase untis (RLU).

Figure 2B and C:B. FACS analysis of MDCK cells infected with H5pp expressing the eGFP reporter gene.

Supernatant of the producer cells were harvested and concentrated 100 times over a sucrose cushion. MDCK were incubated with Δenvpp or different dilutions of H5pp. Incubation of the MDCK cells with a two fold, 16 fold or 256 fold dilutions of the H5pp resulted in 92.3 %, 55,16% or 7 % eGFP-positive cells respectively. (i) FACS analysis (ii) fluorescence images of infected cells. C. EM image of 293T producer cells with budding H5pp. The bar represents 120 nm.

Figure 3: Infectious particle containing secreted H5 protein and p24 proteins can be purified on a sucrose gradient.

Concentrated supernatant was loaded on a 20-60 % sucrose gradient. Fractions were analyzed for (A) infectivity (luciferase activity) (B) HA expression and (C) p24 expression. Infectivity, HA and P24 are detected in the same fractions 10 to 16.

Figure 4A: Different cell types are permissive for H5pp and the entry is dependent on sialic acid.

Cells were pretreated with medium containing 0,025 U/ ml sNA for 1 h at 37 °C. Then, the cells were incubated with concentrated H5pp in the presence or absence of sNA. Infection is expressed in relative luciferase untis (RLU).

Figure 4B: Infection with H5pp depends on expression of α2-3- linked sialic acids.

Huh7 cells were pre-incubated for various time periods with sNA (0.025 U/ml) prior to infection with H5pp. At the time of infection, SNA and MAA staining was performed to determine α2-6- linked and α2-3- linked sialic acid (SA) expression respectively. All data are expressed in percentage compared to the no treatment control, which is considered 100 %.

Figure 5: H5 pp entry is pH-dependent.

Cells were treated with medium containing the indicated concentrations of NH4Cl as described in materials and methods prior to infection with H5pp or AMLVpp. Infectivity was measured by luciferase expression and are expressed as % of control (untreated cells).

Figure 6A: Infection of H5pp, but not VSV-G are neutralized by pre-incubation of H5pp with sera from H5N1 infected patients.

Infectivity of pseudotyped viral particles incubated with indicated dilutions of the sera for 1 h at 37°C is visualized. The yield is expressed in relative luciferase untis (RLU).

Figure 6B: Sera from H5N1 infected persons and vaccinated persons neutralize the infection of H5pp.

Infectivity of pseudotyped viral particles incubated with indicated dilutions of the sera for 1 h at 37°C. The luciferase expression is presented as downregulation compared to no serum control. NIH #1-#5 are sera from H5N1 vaccinated persons.

Figure 6C: Sera from H5N1 infected and vaccinated poultry neutralize the infection of H5pp.

Infectivity of pseudotyped viral particles incubated with indicated dilutions of the sera for 1 h at 37°C. The luciferase expression is presented as downregulation compared to no serum control.

Figures 7A shows a preferred nucleotide sequence of an hemagglutinin used according to the present invention and set forth as SEQ ID NO 1.

Optimised H5 P0408008 (A/Cambodia/2005) from a H5N1 infected patient from Cambodia. Negative cis-acting motifs have been removed and codon as well as signal peptide optimised.

Figures 7B shows a preferred nucleotide sequence of an hemagglutinin used according to the present invention and set forth as SEQ ID NO 2.

Optimised sequence CAC86622 (A/New Caledonia/20/99(H1N1). Negative cis-acting motifs have been removed and codon as well as signal peptide optimised.

Figures 7C shows a preferred nucleotide sequence of an hemagglutinin used according to the present invention and set forth as SEQ ID NO 3.

Optimised sequence of H3N2 5504. Negative cis-acting motifs have been removed and codon as well as signal peptide optimised.

Figures 7D shows a preferred nucleotide sequence of an hemagglutinin used according to the present invention and set forth as SEQ ID NO 4.

Optimised sequence of H7N7 Netherlands. Negative cis-acting motifs have been removed and codon as well as signal peptide optimised.

Figures 7E shows a preferred nucleotide sequence of an hemagglutinin used according to the present invention and set forth as SEQ ID NO 5.

Optimised sequence of H5 Indonesia. Negative cis-acting motifs have been removed and codon as well as signal peptide optimised.

DETAILED DESCRIPTION OF THE INVENTION

Pseudotyped lentiviral particles expressing heterologous viral glycoproteins have been described for several viruses including Hepatitis C (Bartosch, et al., 2003), SARS (Simmons et al., 2004) and the avian influenza virus H7N7 (Duisit et al., 2002). The principle of production is schematically presented in Figure 1A. H5 pseudotyped particles (H5pp) preferably contemplated by the present invention are capable only of a single-round infection and do not produce progeny virus, and can therefore be produced under lower biosafety requirements than the wild-type virus.

The inventors have thus developed a system to produce lentiviral particles pseudotyped with an hemagglutinin, preferably H5 (H5pp) isolated from a patient in Cambodia. H5pp show similar entry characteristics with respect to receptor usage, pH requirement and neutralization compared to the wild type H5N1. The system described herein finds a particular use in serodiagnostic assays and analysis of cellular pathways of influenza entry, and more particularly for H5N1 subtype.

It is therefore an embodiment of the present invention to use an hemagglutinin pseudotyped virus as described further below for the detection of Influenza antibodies. In this connection, the present invention provides a method for detecting the presence or absence in a sample of Influenza antibodies.

The detecting method of the present invention comprises the following steps:

a) contacting the sample with an hemagglutinin pseudotyped virus under conditions sufficient to form an immune complex; and

b) detecting the presence or absence of the immune complex formed in a).

It will be understood that the method of the invention may detect non-neutralizing and/or neutralizing antibodies with respect to Influenza. In a case where the detection of Influenza neutralizing antibodies are preferred, the present method of the invention may consist of a neutralizing assay, such as the one described in the Examples Section. Alternatively, if anti-hemagglutinin antibodies in general have to be detected, the present method of the invention may take the form of an ELISA for instance. Of course, one skilled in the art will appreciate that the present method of the invention may also consist of any other suitable type of assay for the detection of anti-Influenza antibodies.

It will be further understood that by "immune complex" it is meant that the antibodies in the sample bound, in a specific manner, to the hemagglutinin.

As used herein, the term "sample" refers to a variety of sample types obtained from an individual and can be used in the method of the invention. The definition encompasses blood and any other liquid samples of biological origin which may be suspected of containing anti-Influenza antibodies. Preferably, the sample consists of a blood sample from a subject selected from the group consisting of a human, an avian and any animal subject of being infected by an influenza virus, and preferably of a H5N1 subtype.

As used therein, the expression "under condition sufficient to form an immune complex" refers to the conditions in terms of time and temperature for instance that are used to allow antibodies in the sample to specifically bind to the hemagglutinin of the pseudotyped virus. For instance, conditions that are preferably contemplated in a neutralizing assay are shown in the Examples Section.

A preferred hemagglutinin pseudotyped virus contemplated by the present invention consists of a viral expressing vector. For instance, such a contemplated hemagglutinin pseudotyped virus consists of an isolated and purified hemagglutinin pseudotyped virus, such as an hemagglutinin pseudotyped lentivirus. More

particularly, the isolated and purified hemagglutinin pseudotyped virus comprises an hemagglutinin which is encoded by :

- a polynucleotide encoding an H1 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 2;
- a polynucleotide encoding an H3 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 3;
- a polynucleotide encoding an H7 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 4, or
- a polynucleotide encoding an H5 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 1 or 5.

As it may be appreciated, the hemagglutinin sequences preferably contemplated by the present invention have been advantageously optimised in order to eliminate splice site, cryptic splice sites, RNA instability motifs and in order to optimise the codon and signal peptide (Fig 7A-7E).

In accordance with a preferred embodiment, the hemagglutinin is H5 and has a nucleotide sequence as set forth in SEQ ID NO 1 or 5.

As one skilled in the art may appreciate, the hemagglutinin pseudotyped virus contemplated by the present invention advantageously comprises a reporter protein, such as luciferase. Any other reporter gene, such as GFP, that is suitable according to one skilled in the art is within the scope of the present invention.

It will thus be appreciated by one skilled in the art that the method of the present invention finds particular advantageous applications in the serodiagnosis of H5N1 exposed or infected humans and animals in outbreak or epidemic situations, and in the serodiagnosis in large scale seroprevalence in humans and animals to determine the degree of protective immunity against H5N1 in the general population. The methods of the invention such as the H5pp technology requires only BSL2 containment and therefore renders seroneutralisation techniques for H5N1 for instance accessible to countries and institutions which do not have BSL3 laboratories required for microneutralisation. The method of the present invention further finds a

particular advantageous application in the serodiagnosis of large number of sample in HTS format in BSL laboratories.

Yet another advantageous application of the present method consists in the detection of protective H5N1 antibody levels in H5N1 exposed or vaccinated humans, animals and avians.

A further embodiment of the invention consists of the use of an hemagglutinin pseudotyped virus as defined above for the detection of modulators of Influenza virus entry in a cell such as a compound that inhibits or promotes such entry. Indeed, as one skilled in the art will appreciate, the contemplated hemagglutinin pseudotyped virus can be advantageously used in order to identify Influenza entry factors and inhibitors. For instance and as shown in the Examples Section, since the inventors have surprisingly found that, the H5pp specifically reproduces the entry step of H5N1 and it can therefore be used to identify cellular factors (receptors, etc.) that play a role in H5N1 entry. In the same line of thought, H5pp can be used to identify H5N1 entry inhibitors, e.g., small molecule inhibitors.

Therefore, in closely related aspects, the present invention provides methods for identifying a compound that inhibits or promotes the entry of an Influenza virus into a cell; the method comprises the steps of :

a) providing a mixture containing said cells and the compound to be tested;

b) contacting said mixture with an hemagglutinin pseudotyped virus as define above under conditions to allow entry of said virus into the cell; and

c) evaluating the capacity of the compound to inhibit or to promote the entry of the hemagglutinin pseudotyped virus into said cell.

It also provides methods for identifying a compound that promotes or inhibits the entry of an Influenza virus into a cell; the method comprising the steps of :

a) providing a mixture containing said an hemagglutinin pseudotyped virus as defined above and a compound to be tested;

b) adding said mixtures into a cell culture under conditions to allow the entry of said virus into the cells; and

c) evaluating the capacity of the compound to promote or inhibit the entry of the hemagglutinin pseudotyped virus into said cell.

As used herein, the expression "compound that inhibits" refers to a regulatory compound that inhibits, interferes with the entry into a cell of an Influenza virus, such as one of the H5N1 type. A "compound that promotes" as used herein is a regulatory compound that promotes, induces, or facilitates the entry into a cell of an Influenza virus, such as one of the H5N1 type.

The present invention further provides a kit for use within the detection method of the present invention. Such a kit typically comprises two or more components necessary for performing an antibody detection assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain an hemagglutinin pseudotyped virus, such as H5pp, that will allow the binding onto it of a specific antibody such as an anti-H5 antibody. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. In this connection, a further embodiment of the present invention concerns a serodiagnostic kit for the detection of the presence or absence of antibodies indicative of Influenza virus, comprising:

- an hemagglutinin pseudotyped virus as defined above;
- a reagent to detect an hemagglutinin-antibody immune complex;
- optionally a biological reference sample lacking antibodies that immunologically bind with said hemagglutinin; and
- optionally a comparison sample comprising antibodies which can specifically bind to said hemagglutinin;

wherein said hemagglutinin pseudotyped virus, reagent, biological reference sample, and comparison sample are present in an amount sufficient to perform said detection.

With respect to the antibodies of the invention, the expression "specifically

binds to" refers to antibodies that bind to one or more epitopes of an hemagglutinin of interest, but which do not substantially recognize and bind other molecules in a sample containing a mixed population of antigenic biological molecules.

EXAMPLES

Highly pathogenic avian influenza (HPAI) H5N1 has spread globally in birds and infected over 270 humans with an apparently high mortality rate. Serologic studies to determine the extent of asymptomatic H5N1 infection in humans and other mammals and to investigate the immunogenicity of current H5N1 vaccine candidates have been hampered by the biosafety requirements needed for H5N1 micro-neutralization tests. **Objective:** Development of a serodiagnostic tool for highly pathogenic influenza that reproduces H5N1 biology but can be used with less biohazard. **Study Design**: We have generated and evaluated H5 hemagglutinin pseudotyped lentiviral particles encoding the luciferase reporter (H5pp). **Results:** H5pp entry into target cells depends on α2-3 cell surface sialic acids and requires low pH for membrane fusion. H5pp infectivity is specifically neutralized by sera from patients and animals infected with H5N1 and correlates well with conventional microneutralization test. **Conclusions:** H5pp reproduce H5N1 influenza virus entry into target cells and potentially provides a high-throughput and safe method for sero-epidemiology.

Experimental procedures

Production and purification of H5pp

The pseudotyped particles production and sucrose gradient purification were performed as described previously (Lozach et al., 2004). Briefly, HEK293T cells were transfected with pNL Luc E- R- and pCDNA-synH5 (for luciferase) or pcHMWS-EGFP, pCDNA-synH5 and pCMV-dR8.91 (for eGFP) and grown in the presence of soluble *Vibrio cholerae* neuraminidase (sNA) (6,2 mU/ml; Roche). Supernatant was

harvested 24 hours posttransfection, filtered and concentrated and particles were titrated in an infectivity assay using luciferase and the HIV p24 as readout. For sucrose purification, 200 ng of p24 equivalent of H5pp were centrifuged overnight over a 20-60 % discontinuous sucrose gradient. Fractions of 600 µl were taken and pelleted in the presence of 0.05% BSA. The pellet was dissolved in 75 µl DMEM; 1/10 was used to infect MDCK cells and 1/5 was used to perform a western-blot as described (Lozach et al., 2004). Immunostaining was performed with an anti-H5N1 duck serum, a human anti-H5 serum (TH001) or an anti-p24 (Abcam, ab9044).

Neutralization assay

MDCK cells (4000 cells/well) were seeded in 96-well plates in 100 uL of DMEM. 104 RLU of H5pp were incubated with twofold serial dilutions of serum (starting dilution 1:20) for 1 hour at 37°C (CO2 incubator) in 60 uL total DMEM. Subsequently, 100 uL of fresh medium was added and 140 uL of the virus-antibody mixture was transferred to the cells. The luciferase assay was performed 48h later by direct addition of Steady-Glow Luciferase substrate (Promega). Sera were scored positive when they inhibited the H5pp infectivity ≥80% at serum dilutions ≥1/20.

Lectin staining

Huh7 cells were treated with 0.025 U/ml sNA (Roche) for the indicated periods of times. They were harvested by Accutase (Sigma) treatment, washed and distributed over a 96-well plate. Cells were incubated with Fluorescein SNA (Vector laboratories, 10 µg/ml), Biotinylated MAA II (Vector laboratories, 20 µg/ml) or PBS containing 1% BSA (1h, 4°C) and washed three times. When needed, secondary staining was performed with Streptavidin-FITC (BD, 25 µg/ml) prior to analysis by FACScan.

Sera

Human sera from confirmed H5N1 patients were provided by Institut Pasteur Cambodia and the University of Hong Kong. Sera from H5N1 vaccinated volunteers, collected at the 28 day post second dose of the vaccine (Treanor et al., 2006) were provided by Linda Lambert (National Institute of Allergy and Infectious Diseases, Rockville, MD). Avian sera were provided by Institut Pasteur Cambodia and by Robert Webster (St. Jude Children's Research Hospital, Memphis, TN). Serostatus for mammalian sera was confirmed by microneutralization tests and for avian sera by haemagglutination inhibition tests (WHO 2002; OIE 2004).

Example 1: Production of infectious lentiviral H5 pseudotyped particles (H5pp)

The HA of A/Cambodia/408008/05 (H5N1) virus was used. Sequence analysis confirmed that this is a clade 1 H5N1 virus with no known mutations relevant for receptor binding including E190 and G225, suggesting that the H5 protein retained the binding characteristics for α2,3-linked SA. The HA sequence was codon-optimized (Geneart, Germany), cloned into a eukaryotic expression vector and protein expression verified after transfection into HEK293T cells by Western (Figure 1B). Three main bands were seen which is consistent with the expected molecular weight for the uncleaved HA0 and the cleaved subunits HA1 and HA2.

H5pp were generated as described in Figure 1A. Figure 2A shows that H5 pseudotyped but not non-enveloped particles yielded a luciferase signal in MDCK cells. Addition of sNA during the production of H5pp increased the infectivity by more than 1 log (Figure 2A). FACS and microscopy analysis show that H5pp infectivity could also be observed when using a different reporter gene (eGFP) (Figure 2B). Electron microscopy confirmed that viral particles with an average size of 130 nm were generated in transfected cells and could be visualized at the plasma membrane (Figure 2C).

To confirm that the luciferase signal was due to H5pp, concentrated supernatants of producer cell lines were separated over a sucrose gradient and fractions tested for infectivity in MDCK cells and for the presence of HA and p24

antigens. The peak of infectivity, H5 and p24 protein detection was detected in the same fractions 10 to 16 (Figure 3A and 3B).

In addition to MDCK cells Huh7, 293T, BHK, Vero, HeLa and J774 cells were also susceptible to H5pp infection (Figure 4A) which is in accordance with previous reports (Matlin et al., 1981; Kaverin and Klenk, 1995; Kaverin and Webster, 1995, Govorkova et al., 1996; Schultz-Cherry et al., 1998; Kuiken et al., 2003; Rimmelzwaan et al., 2003).

Example 2: H5pp reproduce key steps of H5N1 entry mechanism

Removal of the sialic acids from the cell surface, by pre-incubation of cells with sNA treatment, decreased infection (Figure 4A). *Sambucus Nigra* lectin (SNA) was used to label α2-6-linked SA and *Maackia amurensis* lectin II (MAA) to detect α2-3-linked SA. sNA treatment decreased the expression of both α2-3-linked and α2-6-linked SA, but with different kinetics. Treatment for 30 min diminished the expression of α2-3-linked SA (Figure 4B grey bars), while the α2-6-linked SA were not affected until more prolonged incubation times (Figure 4B black bars). The reduction of the infection upon sNA treatment closely correlates with the expression level of α2-3-linked SA. A 30 min pre-incubation period with sNA resulted in a decrease in infection (Figure 4B white bars) while longer incubation times did not further decrease the levels of infection.

The present results show that infectivity by H5pp, but not AMLVpp was greatly reduced by NH4Cl treatment which inhibits acidification of the endosomes in a dose-dependent manner (Figure 5). Similar results were obtained with Bafilomycin A, another inhibitor of endosomal acidification. Altogether the present results show that H5pp uses α2-3-linked SA as receptor and infect cells in a pH-dependent mechanism.

Example 3: H5pp are neutralized by patient sera

Initial neutralization experiments showed that a convalescent serum from a H5N1 infected patient (TH001) reduced H5pp infectivity to background levels while infectivity of VSV-Gpp was not affected (Figure 6A). A serum taken from a another H5N1 patient (p0302295) at day 10 after the onset of disease was less potent in neutralizing H5pp but still inhibited infectivity of H5pp. These findings were extended by analyzing the neutralizing capacity of additional human and avian sera in the H5pp neutralizing assay (Figure 6 and Table 2).

15/15 sera from confirmed H5N1 patients, 5/5 post-H5N1 human vaccinee sera and 2/227 human sera from healthy controls inhibited H5pp infectivity (sensitivity 100%; specificity 99%) (Table 1A). The two false positive human sera were from persons >65 years of age. Two other human sera (#260, elderly and #195 child) had indeterminate neutralizing activity.

12/12 confirmed H5 seropositive avian sera and 0/41 seronegative avian sera neutralized H5pp. 8/10 sera from H5 vaccinated chicken and ducks tested positive. The two negative sera were also negative in conventional microneutralization tests.

General comments

The inventors have developed lentiviral vectors expressing H5 hemagglutinin (H5pp). H5pp entry requires α2,3-linked SA and low endosomal pH and can be abrogated by sera containing H5N1-specific antibodies. Selective removal of cell surface α2,3-linked SA (Ada, et al., 1961; Tomlinson et al., 1992) downregulates H5pp infection highlighting that H5pp are an ideal tool to study influenza cell tropism such as the role of critical HA residues in receptor usage.H5pp encode a reporter gene which allows for high throughput analyses under lower biosafety requirements than wild type H5N1.

Using a novel H5pp neutralization assay, the inventors detected neutralizing antibodies in sera from previously confirmed avian and human H5N1 cases. The inventors have observed a tight correlation of the H5pp neutralization assay with characterized positive and negative human and avian (Table 1AB). The two false

positive sera were detected in the H5pp assay came from elderly persons and it was previously reported that false positive results in the H5 microneutraliztion test are also more common in the elderly (Rowe et al., 1999). While a more comprehensive clinical and epidemiological evaluation is needed, the results presented herein indicate the H5pp neutralization assay is a novel approach that can be used for large-scale H5 seroprevalence studies on human and animal sera. The advantages over existing techniques for detection of H5N1 neutralizing antibodies include easy and flexible read-out, handling under BSL2 condition and the requirement of small quantities of serum. The system is suited for automated high throughput screening in a 96-well plate or 384-well plate format. The H5pp particles can be produced from synthetic codon optimized DNA without access to the live H5N1 virus or cloned viral genes. The flexibility of the system encourages the development of a multiplex assay where different subtypes or clades of influenza viruses can be screened simultaneously. In conclusion, the method of detecting anti-Influenza antibodies of the present invention is clearly a valuable tool in global efforts to increase the surveillance of influenza viruses in avians, animals and humans.

Table 1A: Overview of the human sera tested in the H5pp neutralization test.

H5N1 Status	# Positive / # Tested
H5N1 confirmed patients	15/15
H5N1 vaccinated volunteers	5/5
Healthy controls - ≤ 18 years	0/106
Healthy controls – Young adults	0/20
Healthy controls - ≥ 65 years	2/121

The criterion for H5pp positive is ≥ 80% reduction of infectivity at a serum dilution of ≥ 1/20

Table 1B: Overview of the avian sera tested in the H5pp neutralization test.

H5N1 status	# positive / # Tested
H5N1 positive sera*	12/12
H5N1 negative sera*	0/41
H5N1 vaccinated chicken or ducks	8/10**

The criterion for H5pp positive is ≥ 80% reduction of infectivity at a serum dilution of ≥ 1/20

* Based on hemagglutination inhibition and microneutralization test

** The two negative sera were also negative on microneutralization tests.

Abbreviations:

HI: hemagglutination inhibition

HA: hemagglutinin

H5: hemagglutinin of H5N1

H5pp: H5 pseudotyped viral particles

AMLV: amphotrophic envelope of the murine leukemia virus

sNA: soluble recombinant neuraminidase from *Vibrio cholerae*

SNA: *Sambucus Nigra* (elderberry) Bark lectin

MAA: *Maackia amurensis* lectin II

HPAI: highly pathogenic avian influenza

SA: sialic acids

References

- Ada, G. L., French, E. L., and Lind, P. E. (1961). Purification and properties of neuraminidase from Vibrio cholerae. *J Gen Microbiol* **24,** 409-25.
- Bartosch, B., Dubuisson, J., and Cosset, F. L. (2003). Infectious hepatitis C virus pseudoparticles containing functional E1-E2 envelope protein complexes. *J Exp Med* **197**(5), 633-42.
- Doms, R. W., Helenius, A., and White, J. (1985). Membrane fusion activity of the influenza virus hemagglutinin. The low pH-induced conformational change. *J Biol Chem* **260**(5), 2973-81.
- Dong, J., Roth, M. G., and Hunter, E. (1992). A chimeric avian retrovirus containing the influenza virus hemagglutinin gene has an expanded host range. *J Virol* **66**(12), 7374-82.
- Duisit, G., Conrath, H., Saleun, S., Folliot, S., Provost, N., Cosset, F. L., Sandrin, V., Moullier, P., and Rolling, F. (2002). Five recombinant simian immunodeficiency virus pseudotypes lead to exclusive transduction of retinal pigmented epithelium in rat. *Mol Ther* **6**(4), 446-54.
- Galen, J. E., Ketley, J. M., Fasano, A., Richardson, S. H., Wasserman, S. S., and Kaper, J. B. (1992). Role of Vibrio cholerae neuraminidase in the function of cholera toxin. *Infect Immun* **60**(2), 406-15.
- Govorkova, E. A., Murti, G., Meignier, B., de Taisne, C., and Webster, R. G. (1996). African green monkey kidney (Vero) cells provide an alternative host cell system for influenza A and B viruses. *J Virol* **70**(8), 5519-24.
- Katz, J. M., Lim, W., Bridges, C. B., Rowe, T., Hu-Primmer, J., Lu, X., Abernathy, R. A., Clarke, M., Conn, L., Kwong, H., Lee, M., Au, G., Ho, Y. Y., Mak, K. H., Cox, N. J., and Fukuda, K. (1999). Antibody response in individuals infected with avian influenza A (H5N1) viruses and detection of anti-H5 antibody among household and social contacts. *J Infect Dis* **180**(6), 1763-70.
- Kaverin, N. V., and Klenk, H. D. (1995). The effect of cell density on influenza virus replication in CV-1 cells. *Mol Gen Mikrobiol Virusol*(2), 26-33.

- Kaverin, N. V., Rudneva, I. A., Ilyushina, N. A., Varich, N. L., Lipatov, A. S., Smirnov, Y. A., Govorkova, E. A., Gitelman, A. K., Lvov, D. K., and Webster, R. G. (2002). Structure of antigenic sites on the haemagglutinin molecule of H5 avian influenza virus and phenotypic variation of escape mutants. *J Gen Virol* **83**(Pt 10), 2497-505.
- Kaverin, N. V., and Webster, R. G. (1995). Impairment of multicycle influenza virus growth in Vero (WHO) cells by loss of trypsin activity. *J Virol* **69**(4), 2700-3.
- Kuiken, T., Rimmelzwaan, G. F., Van Amerongen, G., and Osterhaus, A. D. (2003). Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (Macaca fascicularis). *Vet Pathol* **40**(3), 304-10.
- Lin, A. H., and Cannon, P. M. (2002). Use of pseudotyped retroviral vectors to analyze the receptor-binding pocket of hemagglutinin from a pathogenic avian influenza A virus (H7 subtype). *Virus Res* **83**(1-2), 43-56.
- Lozach, P. Y., Amara, A., Bartosch, B., Virelizier, J. L., Arenzana-Seisdedos, F., Cosset, F. L., and Altmeyer, R. (2004). C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem* **279**(31), 32035-45.
- Matlin, K. S., Reggio, H., Helenius, A., and Simons, K. (1981). Infectious entry pathway of influenza virus in a canine kidney cell line. *J Cell Biol* **91**(3 Pt 1), 601-13.
- OIE (2004), Manual of diagnostic tests and vaccines for terrestrial animals, http://www.oie.int/eng/normes/mmanual/A_summry.htm
- Philpott, M., Easterday, B. C., and Hinshaw, V. S. (1989). Neutralizing epitopes of the H5 hemagglutinin from a virulent avian influenza virus and their relationship to pathogenicity. *J Virol* **63**(8), 3453-8.
- Rimmelzwaan, G. F., Kuiken, T., van Amerongen, G., Bestebroer, T. M., Fouchier, R. A., and Osterhaus, A. D. (2003). A primate model to study the pathogenesis of influenza A (H5N1) virus infection. *Avian Dis* **47**(3 Suppl), 931-3.

- Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., and Wiley, D. C. (1983). Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. *Nature* **304**(5921), 76-8.
- Rowe, T., Abernathy, R. A., Hu-Primmer, J., Thompson, W. W., Lu, X., Lim, W., Fukuda, K., Cox, N. J., and Katz, J. M. (1999). Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* **37**(4), 937-43.
- Russell, R. J., Stevens, D. J., Haire, L. F., Gamblin, S. J., and Skehel, J. J. (2006). Avian and human receptor binding by hemagglutinins of influenza A viruses. *Glycoconj J* **23**(1-2), 85-92.
- Schultz-Cherry, S., Dybdahl-Sissoko, N., McGregor, M., and Hinshaw, V. S. (1998). Mink lung epithelial cells: unique cell line that supports influenza A and B virus replication. *J Clin Microbiol* **36**(12), 3718-20.
- Simmons, G., Reeves, J. D., Rennekamp, A. J., Amberg, S. M., Piefer, A. J., and Bates, P. (2004). Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. *Proc Natl Acad Sci U S A* **101**(12), 4240-5.
- Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., and Wiley, D. C. (1982). Changes in the conformation of influenza virus hemagglutinin at the pH optimum of virus-mediated membrane fusion. *Proc Natl Acad Sci U S A* **79**(4), 968-72.
- Skehel, J. J., Daniels, R. S., Douglas, A. R., and Wiley, D. C. (1983). Antigenic and amino acid sequence variations in the haemagglutinins of type A influenza viruses recently isolated from human subjects. *Bull World Health Organ* **61**(4), 671-6.
- Skehel, J. J., and Wiley, D. C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. *Annu Rev Biochem* **69**, 531-69.
- Skehel, J. J., and Wiley, D. C. (2002). Influenza haemagglutinin. *Vaccine* **20 Suppl 2**, S51-4.

- Stephenson, I., Wood, J. M., Nicholson, K. G., and Zambon, M. C. (2003). Sialic acid receptor specificity on erythrocytes affects detection of antibody to avian influenza haemagglutinin. *J Med Virol* **70**(3), 391-8.
- Tomlinson, S., Pontes de Carvalho, L., Vandekerckhove, F., and Nussenzweig, V. (1992). Resialylation of sialidase-treated sheep and human erythrocytes by Trypanosoma cruzi trans-sialidase: restoration of complement resistance of desialylated sheep erythrocytes. *Glycobiology* **2**(6), 549-51.
- Treanor, J. J., Campbell, J. D., Zangwill, K. M., Rowe, T., and Wolff, M. (2006). Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *N Engl J Med* **354**(13), 1343-51.
- Vong, S., Coghlan, B., Mardy, S., Holl, D., Seng, H., Ly, S., Miller, M., Buchy, P., Froehlich, Y., Dufourcq, J., Uyeki, T., Lim, W., and Sok, T. (2006). Low Frequency of Poultryto-Human H5N1 Virus Transmission, Southern Cambodia, 2005. *Emerging Infectious diseases* **12**(10).
- Wiley, D. C., and Skehel, J. J. (1987). The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. *Annu Rev Biochem* **56**, 365-94.
- WHO (2002), WHO Manual on animal influenza diagnosis and surveillance, http://www.wpro.who.int/NR/rdonlyres/EFD2B9A7-2265-4AD0-BC98-97937B4FA83C/0/manualonanimalaidiagnosisandsurveillance.pdf
- Yoshimura, A., Kuroda, K., Kawasaki, K., Yamashina, S., Maeda, T., and Ohnishi, S. (1982). Infectious cell entry mechanism of influenza virus. *J Virol* **43**(1), 284-93.

WHAT IS CLAIMED IS:

1. A method for detecting the presence or absence of Influenza antibodies in a sample, comprising the steps of
   a) contacting the sample with an hemagglutinin pseudotyped virus under conditions sufficient to form an immune complex; and
   b) detecting the presence or absence of the immune complex formed in a).

2. The method according to claim 1, wherein the sample consists of a blood sample from a subject selected from the group consisting of a human, an animal and an avian.

3. The method according to claim 1 or 2, wherein the antibodies consist of neutralizing antibodies.

4. The method according to any one of claims 1 to 3, wherein the virus consists of a viral expressing vector.

5. The method according to claim 4, wherein the virus consists of a lentivirus.

6. The method according to claim 1, wherein the hemagglutinin consists of an hemagglutinin selected from the group consisting of H1, H3, H5 and H7.

7. The method of claim 3, wherein the hemagglutinin comprises a nucleotide sequence selected from the group consisting of SEQ ID NO 1, 2, 3, 4 and 5.

8. The method according to claim 6, wherein the hemagglutinin comprises a nucleotide sequence having SEQ ID NO 1 or 5.

9. Isolated and purified hemagglutinin pseudotyped virus, wherein said hemagglutinin is encoded by :
   - a polynucleotide encoding an H1 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 2;
   - a polynucleotide encoding an H3 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 3;
   - a polynucleotide encoding an H7 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 4 or
   - a polynucleotide encoding an H5 hemagglutinin having a nucleotide sequence as set forth in SEQ ID NO 1 or 5.

10. The hemagglutinin pseudotyped virus as defined in claim 9, further comprising a reporter protein.

11. The hemagglutinin pseudotyped virus as defined in claim 10, wherein said reporter protein is luciferase or GFP.

12. Use of an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 for the detection of Influenza antibodies.

13. The use according to claim 12, wherein the virus is a lentivirus.

14. The use according to claim 12 or 13, wherein the hemagglutinin consists of H5.

15. Use of an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 for the detection of a compound that inhibits the entry of an Influenza virus in a cell.

16. The use according to claim 15, wherein the virus is a lentivirus.

17. The use according to claim 15 or 16, wherein the hemagglutinin consists of H5.

18. Use of an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 for the detection of a compound that promotes the entry of an Influenza virus in a cell.

19. The use according to claim 18, wherein the virus is a lentivirus.

20. The use according to claim 18 or 19, wherein the hemagglutinin consists of H5.

21. A method for identifying a compound that inhibits the entry of an Influenza virus into a cell; the method comprises the steps of :

a) providing a mixture containing said cells and the compound to be tested;

b) contacting said mixture with an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 under conditions to allow the entry of said virus into the cell; and

c) evaluating the capacity of the compound to inhibit the entry of the hemagglutinin pseudotyped virus into said cell.

22. A method for identifying a compound that promotes the entry of an Influenza virus into a cell; the method comprising the steps of :

a) providing a mixture containing said cells and the compound to be tested;

b) contacting said mixture with an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 under conditions to allow the entry of said virus into the cell; and

c) evaluating the capacity of the compound to promote the entry of the hemagglutinin pseudotyped virus into said cell

23. A method for identifying a compound that inhibits the entry of an Influenza virus into a cell; the method comprises the steps of :

a) providing a mixture containing an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 and a compound to be tested;

b) adding said mixtures into a cell culture under conditions to allow the entry of said virus into the cells; and

c) evaluating the capacity of the compound to inhibit the entry of the hemagglutinin pseudotyped virus into said cell.

24. A method for identifying a compound that promotes the entry of an Influenza virus into a cell; the method comprises the steps of :

a) providing a mixture containing an hemagglutinin pseudotyped virus as defined in any one of claims 9 to 11 and a compound to be tested;

b) adding said mixtures into a cell culture under conditions to allow the entry of said virus into the cells; and

c) evaluating the capacity of the compound to promote the entry of the hemagglutinin pseudotyped virus into said cell.

25. A serodiagnostic kit for the detection of the presence or absence of antibodies indicative of Influenza virus, comprising:

- an hemagglutinin pseudotyped virus according to any one of claims 9 to 11;
- a reagent to detect an hemagglutinin-antibody immune complex;

- optionally a biological reference sample lacking antibodies that immunologically bind with said hemagglutinin; and
- optionally a comparison sample comprising antibodies which can specifically bind to said hemagglutinin;

wherein said hemagglutinin pseudotyped virus, reagent, biological reference sample, and comparison sample are present in an amount sufficient to perform said detection.

26. The kit according to claim 25, wherein the hemagglutinin consists of H5.

27. The kit according to claim 25 or 26, wherein the antibodies consist of neutralizing antibodies.

FIGURE 1A

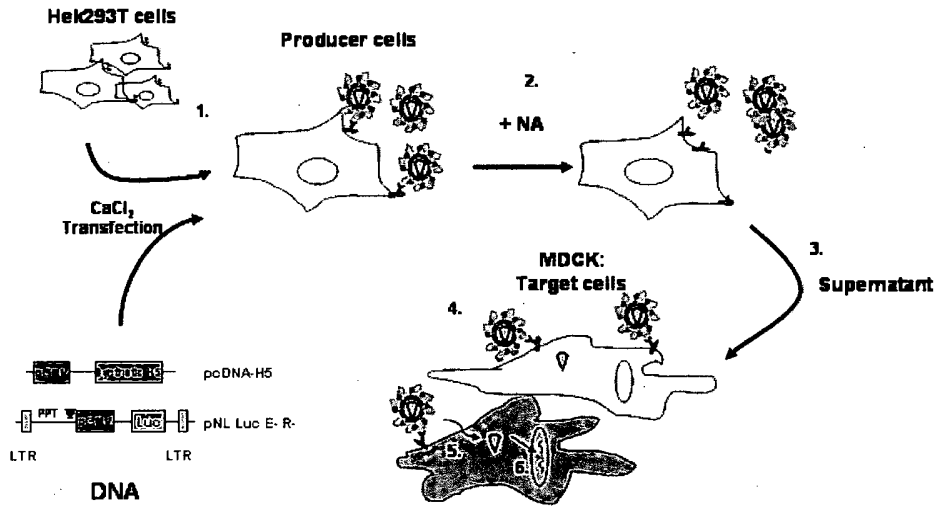
Hek293T cells
Producer cells
1.
2.
+ NA
CaCl2
Transfection
3.
Supernatant
MDCK:
Target cells
4.
pcDNA-H5
pNL Luc E- R-
PPT
Luc
LTR
LTR
DNA

FIGURE 1B

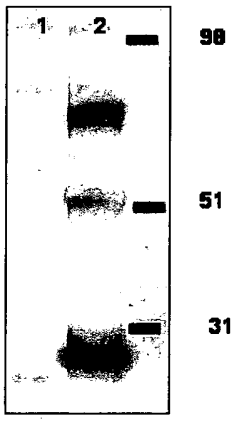

FIGURE 2A

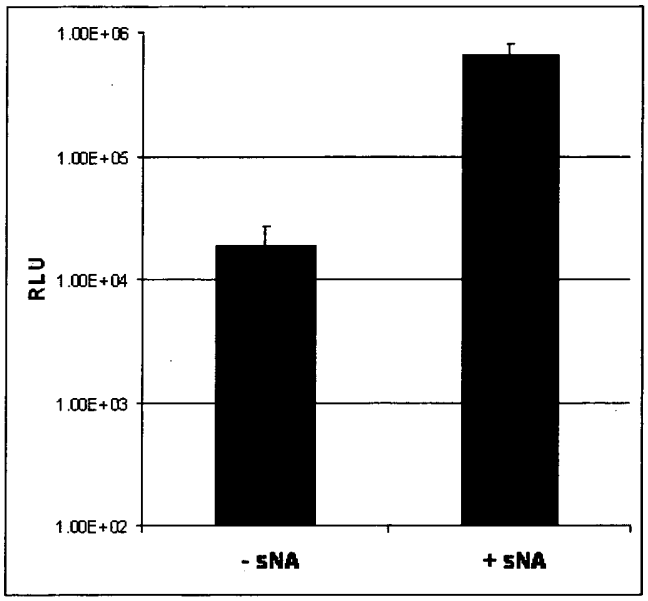

FIGURE 2B

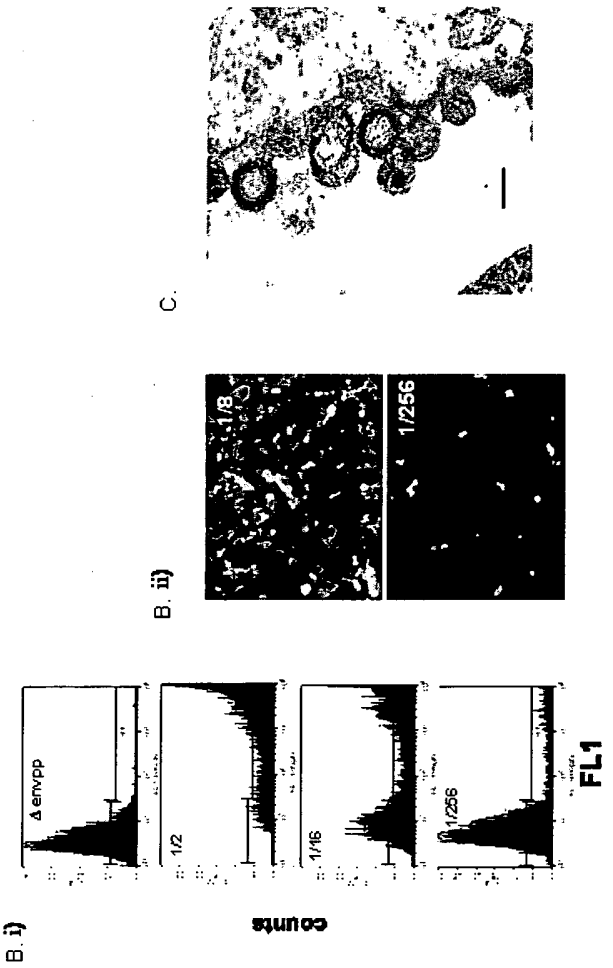

FIGURE 3

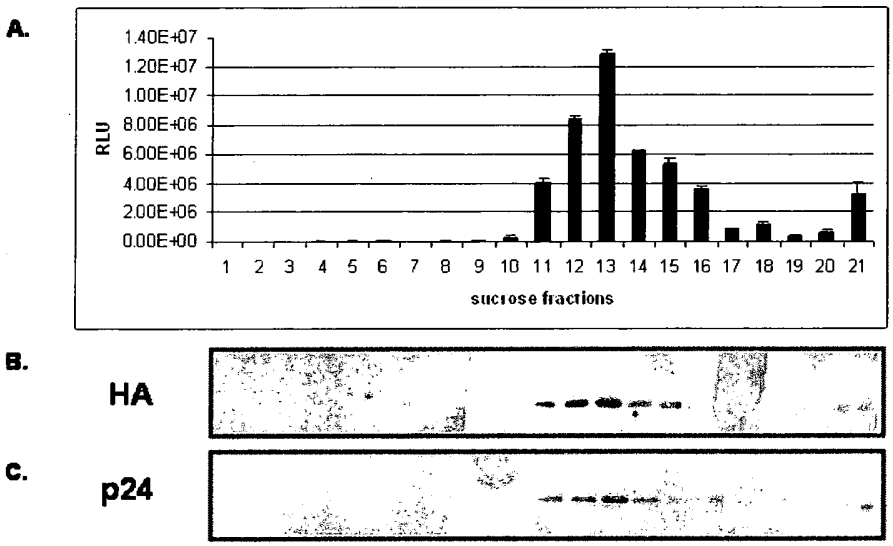
A.
1.40E+07
1.20E+07
1.00E+07
8.00E+06
6.00E+06
4.00E+06
2.00E+06
0.00E+00
RLU
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
sucrose fractions
B.
HA
C.
p24

FIGURE 4A

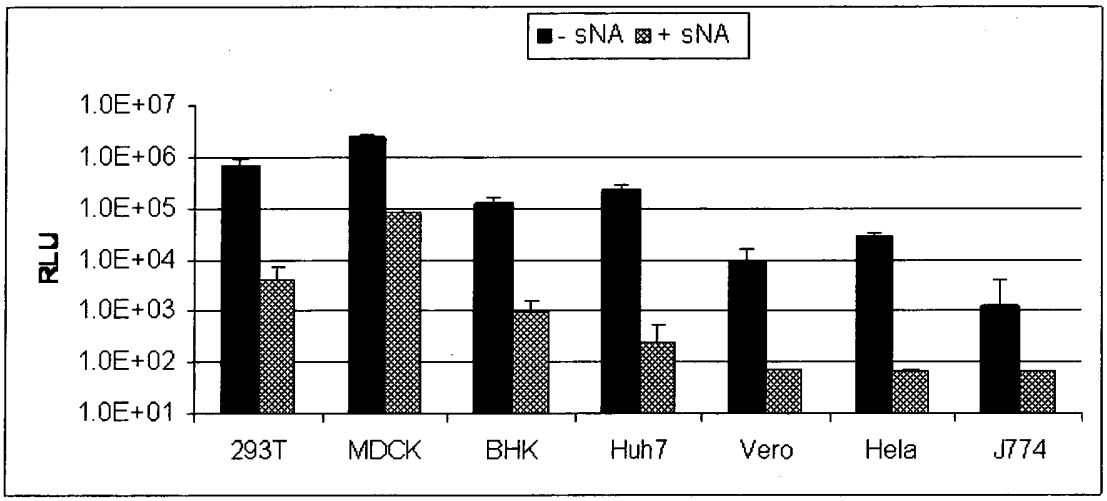
■- sNA ▨+ sNA
RLU
1.0E+07
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
293T
MDCK
BHK
Huh7
Vero
Hela
J774

FIGURE 4B

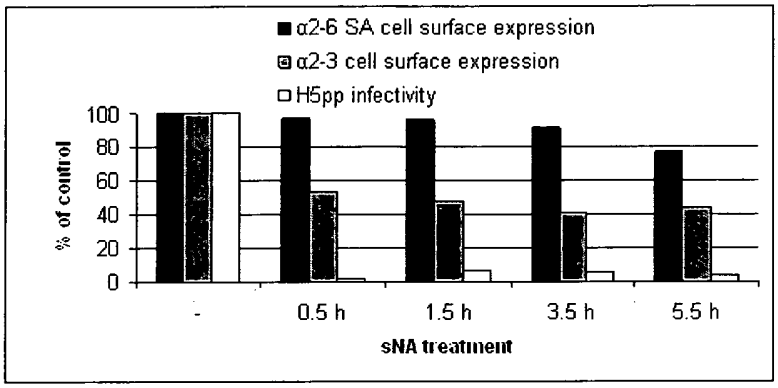
α2-6 SA cell surface expression
α2-3 cell surface expression
H5pp infectivity
% of control
100
80
60
40
20
0
-
0.5 h
1.5 h
3.5 h
5.5 h
sNA treatment

FIGURE 5

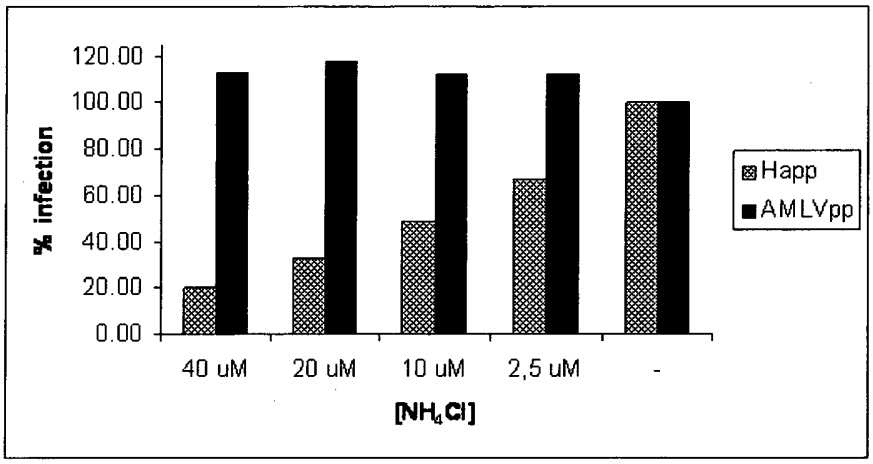
120.00
100.00
80.00
60.00
40.00
20.00
0.00
% infection
40 uM
20 uM
10 uM
2,5 uM
-
[NH4Cl]
Happ
AMLVpp

FIGURE 6A

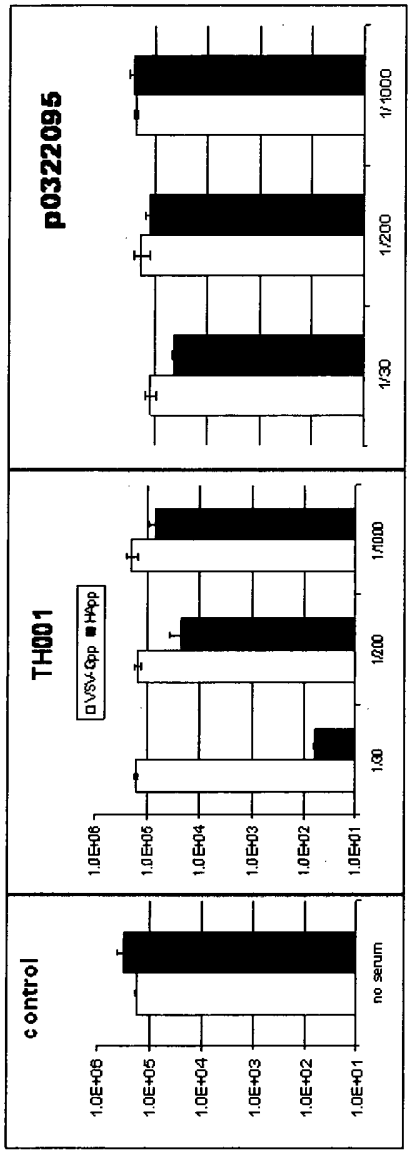
control
TH001
p0322095
VSV-Gpp
HApp
1.0E+06
1.0E+05
1.0E+04
1.0E+03
1.0E+02
1.0E+01
no serum
1/30
1/200
1/1000

FIGURE 6B

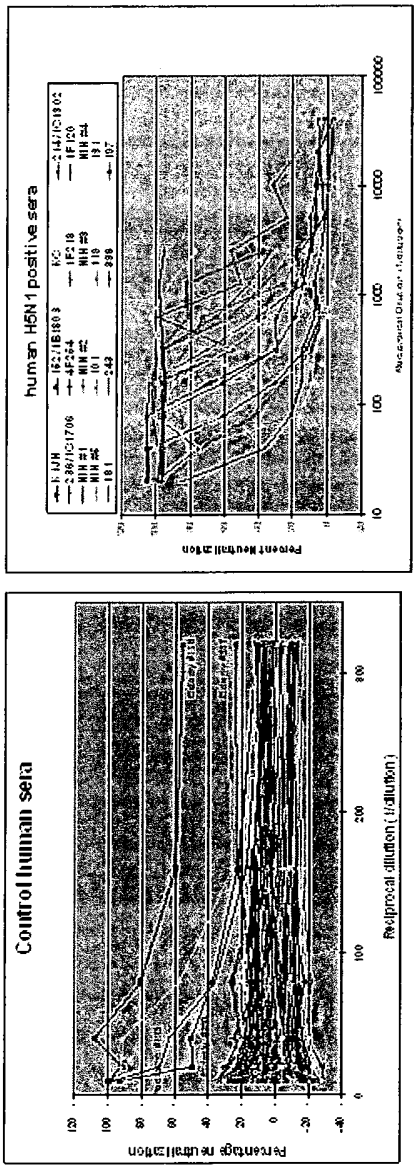
human H5N1 positive sera
Percent Neutralization
Control human sera
Percentage neutralization
Reciprocal dilution (1/dilution)

FIGURE 6C

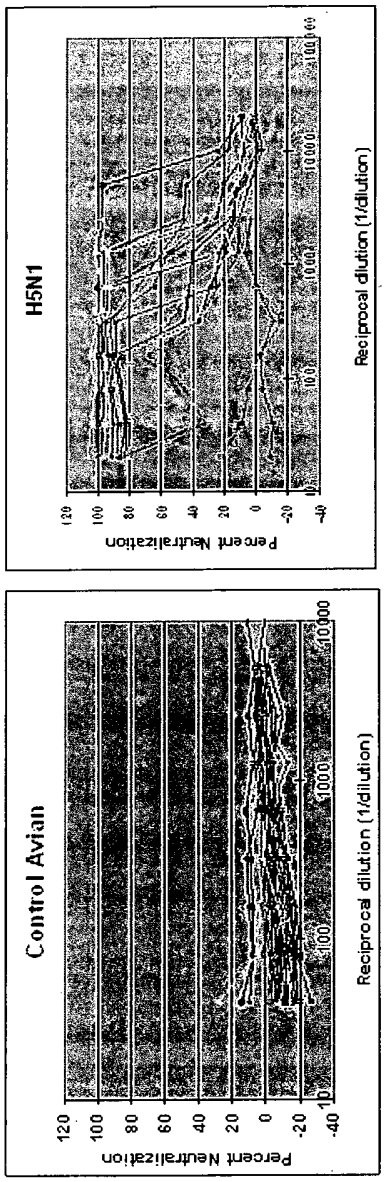
Control Avian
Percent Neutralization
120
100
80
60
40
20
0
-20
-40
10
100
1000
10000
Reciprocal dilution (1/dilution)
H5N1
Percent Neutralization
Reciprocal dilution (1/dilution)

FIGURE 7A

GCGCGCGCGGCCGCCACCATGGAGAAGATCGTGCTGCTGTTCGCCATCGTGTCCCTGGTGAAGA
GCGACCAGATCTGTATCGGCTACCACGCCAACAATAGCACCGAGCAGGTGGACACCATCATGGAG
CGGAACGTGACCGTGACCCACGCCCAGGATATTCTGGAGAAAACCCACAACGGCAAGCTGTGTGA
CCTGGACGGCGTGAAGCCTCTGATCCTGAGGGATTGTAGCGTGGCCGGCTGGCTGCTGGGCAAC
CCCATGTGTGACGAGTTCATCAACGTGCCCGAGTGGAGCTACATCGTGGAGAAGGCCAACCCCGT
GAACGATCTGTGCTACCCCGGCGACTTCAACGATTACGAGGAGCTGAAGCACCTGCTGTCCCGGA
TCAACCACTTCGAGAAGATCCAGATCATCCCCAAGAGCAGCTGGAGCAGCCACGAGGCCAGCCTG
GGCGTGTCCGCCGTGTGTCCCTACCAGGGCAAGTCCAGCTTCTTCCGGAACGTTGTGTGGCTGAT
CAAGAAGAACAGCACCTACCCCACCATCAAGCGGAGCTACAACAACACCAACCAAGAGGACCTGC
TGGTGATGTGGGGCATCCACCACCCCAACGACGCCGCCGAGCAGACCAAGCTGTACCAGAACCC
CACCACCTACATCTCTGTGGGCACCTCCACCCTGAATCAGAGGCTGGTCCCTAGAATCGCCACCA
GGTCCAAGGTGAACGGCCAGAGCGGCAGGATGGAGTTCTTCTGGACCATCCTGAAGCCCAACGA
TGCCATCAACTTCGAGAGCAACGGCAACTTCATCGCCCCTGAGTACGCCTACAAGATCGTGAAGA
AGGGCGACAGCACCATCATGAAGTCCGAGCTGGAGTACGGCAACTGTAACACCAAGTGCCAGAC
CCCCATGGGCGCCATCAATAGCAGCATGCCCTTCCACAACATCCACCCCCTGACCATCGGCGAGT
GCCCCAAGTACGTGAAGAGCAACAGGCTGGTGCTGGCCACCGGCCTGAGAAATAGCCCCCAGCG
GGAGCGGAGAAGAAAGAAGAGGGGCCTGTTCGGCGCCATCGCCGGCTTCATCGAGGGCGGCTG
GCAGGGCATGGTGGACGGCTGGTACGGCTACCACCACAGCAACGAGCAGGGCAGCGGCTACGC
CGCCGACAAGGAGAGCACCCAGAAGGCCATCGACGGCGTCACCAACAAGGTGAACAGCATCATC
GACAAGATGAACACCCAGTTCGAGGCTGTGGGCAGGGAGTTCAACAACCTGGAGCGGCGGATCG
AGAACCTGAACAAGAAGATGGAGGACGGCTTCCTGGATGTGTGGACCTACAACGCCGAGCTGCTG
GTGCTGATGGAGAACGAGCGGACCCTGGACTTCCACGACAGCAACGTGAAGAACCTGTACGACAA
AGTGAGGCTGCAGCTGAGGGACAATGCCAAGGAGCTGGGCAACGGCTGCTTCGAGTTCTACCAC
AAGTGTGACAACGAGTGTATGGAGTCTGTGAGGAACGGCACCTACGACTACCCTCAGTACAGCGA
GGAGGCCAGGCTGAAGAGAGAGGAGATCAGCGGGGTCAAGCTGGAGAGCATCGGCATCTACCAG
ATCCTGAGCATCTACAGCACCGTGGCCAGCAGCCTGGCCCTGGCCATCATGGTGGCCGGCCTGA
GCCTGTGGATGTGTAGCAACGGCAGCCTGCAGTGCAGGATCTGTATT

FIGURE 7B

GGTACCGCGGCCGCGCGCCACCATGAAGGCCAAGCTGCTGGTCCTCCTGTGTACATTCAC
CGCCACCTACGCCGACACCATCTGTATCGGCTACCACGCCAACAACAGCACCGACACCGT
GGATACCGTGCTGGAGAAGAACGTGACCGTGACCCACAGCGTGAACCTGCTGGAGGACAG
CCACAATGGCAAGCTGTGCCTGCTGAAAGGCATCGCCCCCCTGCAGCTGGGCAACTGTAG
CGTGGCCGGCTGGATCCTGGGCAATCCCGAGTGTGAGCTGCTGATCTCCAAGGAGAGCTG
GAGCTACATCGTGGAGACCCCCAACCCCGAGAACGGCACCTGCTACCCCGGCTACTTCGC
CGACTACGAGGAGCTGAGAGAGCAGCTGTCCTCTGTCTCCAGCTTCGAGCGCTTCGAGAT
CTTCCCCAAGGAGTCCAGCTGGCCCAACCACACCGTGACCGGCGTGTCCGCCAGCTGTAG
CCACAACGGGAAGAGCAGCTTCTACCGGAACCTGCTGTGGCTGACCGGCAAGAATGGCCT
GTACCCCAACCTGAGCAAGAGCTACGTGAACAACAAGGAAAAGGAAGTGCTGGTGCTGTG
GGGCGTGCACCACCCCCCCAACATCGGCGACCAGAGAGCCCTGTACCACACCGAGAACGC
CTATGTGAGCGTGGTGTCCAGCCACTACAGCAGGAGATTCACCCCCGAGATCGCCAAGAG
GCCCAAAGTGAGAGACCAGGAGGGCCGGATCAACTACTACTGGACCCTGCTGGAGCCCGG
CGATACCATCATCTTCGAGGCCAACGGCAATCTGATCGCCCCTTGGTACGCCTTCGCCCT
GAGCAGAGGCTTCGGCAGCGGCATCATCACCAGCAACGCCCCCATGGATGAGTGTGACGC
CAAGTGCCAGACCCCCCAGGGCGCCATCAACAGCAGCCTGCCCTTCCAGAATGTGCACCC
CGTGACCATCGGCGAGTGCCCCAAGTATGTGAGGAGCGCCAAGCTGAGAATGGTGACCGG
CCTGAGGAACATCCCTAGCATCCAGAGCAGAGGCCTGTTCGGCGCCATCGCCGGCTTCAT
CGAGGGCGGCTGGACCGGCATGGTGGACGGCTGGTACGGCTACCACCACCAGAACGAGCA
GGGCAGCGGCTACGCCGCCGACCAGAAGAGCACCCAGAACGCCATCAACGGCATCACCAA
CAAGGTGAACAGCGTGATCGAGAAGATGAACACCCAGTTCACCGCTGTGGGCAAGGAGTT
CAACAAGCTGGAGCGGCGGATGGAGAACCTGAACAAGAAGGTGGACGACGGCTTCCTGGA
CATCTGGACCTACAACGCCGAACTGCTGGTCCTGCTGGAGAACGAGAGGACCCTGGACTT
CCACGACAGCAACGTGAAGAACCTGTATGAGAAGGTGAAGAGCCAGCTGAAGAACAACGC
CAAGGAGATCGGCAACGGCTGCTTCGAGTTCTACCACAAGTGTAACAACGAGTGTATGGA
GAGCGTGAAGAACGGGACCTACGACTACCCCAAGTACAGCGAGGAGAGCAAGCTGAACCG
GGAGAAGATCGACGGCGTGAAGCTGGAGAGCATGGGCGTGTACCAGATCCTGGCCATCTA
CAGCACCGTGGCCAGCAGCCTGGTGCTGCTGGTGTCCCTGGGCGCCATCTCCTTTTGGAT
GTGCTCCAACGGCAGCCTGCAGTGCAGGATCTGCTGATGACCGGTTAACTCGAGCTC

FIGURE 7C

GGTACCGCGGCCGCGCGCCACCATGAAAACCATCATCGCCCTGAGCTACATCCTGTGCCT
GGTGTTCGCCCAGAAGCTGCCCGGCAACGATAACAGCACCGCCACCCTGTGTCTGGGCCA
CCACGCCGTGCCCAACGGCACCATCGTGAAAACAATCACCAACGACCAGATCGAGGTGAC
CAATGCCACCGAGCTGGTGCAGATCAGCAGCACCGGCGGCATCTGTGACAGCCCTCACCA
GATCCTGGACGGCGAGAACTGTACCCTGATCGATGCCCTGCTGGGCGATCCTCAGTGTGA
CGGCTTCCAGAACAAGAAGTGGGACCTGTTCGTCGAGAGGAGCAAGGCCTACAGCAACTG
CTACCCCTACGACGTGCCTGACTACGCCAGCCTGAGAAGCCTGGTGGCCAGCAGCGGCAC
CCTGGAGTTCAACAACGAGAGCTTCGACTGGACCGGCGTGACCCAGAACGGCACCAGCAG
CGCCTGTAAGCGGAGGAGCAACAAGAGCTTCTTCTCCCGGCTGAACTGGCTGACCCACCT
GAAGTACAAGTACCCCGCCCTGAACGTGACCATGCCCAACAATGAGAAGTTCGACAAGCT
GTACATCTGGGGCGTGCACCACCCCGGCACCGACAGCGACCAGATCAGCCTGTACGCCCA
GGCCAGCGGCAGAATCACAGTGAGCACCAAGAGGAGCCAGCAGACCGTGATCCCCAACAT
CGGCAGCAGACCCAGAGTGAGGGACGTGTCCAGCCGGATCAGCATCTACTGGACAATCGT
GAAGCCCGGCGACATCCTGCTGATCAACTCCACCGGCAACCTGATCGCCCCTCGGGGCTA
CTTCAAGATCCGGAGCGGCAAGAGCAGCATCATGAGAAGCGACGCCCCTATCGGCAAGTG
TAACAGCGAGTGTATCACCCCCAATGGCAGCATCCCCAACGACAAGCCCTTCCAGAACGT
GAACCGGATCACCTACGGCGCCTGCCCTAGATACGTGAAGCAGAACACCCTGAAGCTGGC
CACCGGCATGAGAAACGTGCCCGAGAAGCAGACCAGAGGCATCTTCGGCGCCATCGCCGG
CTTCATCGAGAACGGCTGGGAGGGCATGGTGGACGGCTGGTACGGCTTCAGACACCAGAA
TAGCGAGGGCACCGGCCAGGCCGCCGACCTGAAGAGCACCCAGGCCGCCATCAACCAGAT
CAACGGCAAGCTGAACCGGCTGATCGGCAAGACCAACGAGAAGTTCCACCAGATCGAAAA
GGAATTCAGCGAGGTGGAGGGCAGAATCCAGGACCTGGAGAAGTACGTGGAGGACACCAA
GATCGACCTGTGGAGCTACAATGCCGAGCTGCTGGTCGCCCTGGAGAATCAGCACACCAT
CGACCTGACCGACTCCGAGATGAACAAGCTGTTCGAGCGGACCAAGAAGCAGCTGAGGGA
GAACGCCGAGGATATGGGCAACGGCTGCTTTAAGATCTACCACAAGTGTGACAACGCCTG
TATCGGCAGCATCCGGAACGGCACCTACGACCACGACGTGTACAGAGATGAGGCCCTGAA
CAACCGCTTCCAGATCAAGGGCGTGGAGCTGAAGAGCGGCTACAAGGACTGGATCCTGTG
GATCAGCTTCGCCATCAGCTGCTTTCTGCTGTGTGTGGCCCTGCTGGGATTCATCATGTG
GGCCTGCCAGAAGGGCAACATCCGCTGTAACATCTGTATCGGCGGCGACTACAAGGACGA
CGACGATAAGTGATGACCGGTTAACTCGAGCTC

FIGURE 7D

GGTACCGCGGCCGCGCGCCACCATGAGCAAGAGCCGGGGCTACAAGATGAACACCCAGAT
CCTGGTGTTCGCCCTGGTGGCCAGCATCCCTACCAACGCCGACAAGATCTGCCTGGGCCA
CCACGCCGTGTCCAACGGCACCAAGGTGAACACCCTGACCGAGAGGGGCGTGGAGGTGGT
GAACGCCACCGAGACCGTGGAGAGAACCAACGTGCCCCGGATCTGTAGCAAGGGCAAGAG
GACCGTGGACCTGGGCCAGTGTGGCCTGCTGGGCACCATCACCGGCCCTCCTCAGTGTGA
TCAGTTCCTGGAGTTCAGCGCCGACCTGATCATCGAGAGGAGAGAAGGCAGCGATGTGTG
CTACCCCGGCAAGTTCGTGAACGAGGAGGCCCTGAGGCAGATCCTGAGAGAGAGCGGCGG
CATCGACAAGGAAACCATGGGCTTCACCTACAGCGGCATCAGGACCAATGGCACCACCTC
CGCCTGTAGAAGAAGCGGCAGCAGCTTCTACGCCGAGATGAAGTGGCTGCTGTCCAACAC
CGACAATGCCGCCTTCCCCCAGATGACCAAGAGCTACAAGAACACCAGGAAGGACCCCGC
CCTGATCATCTGGGGCATCCACCACAGCGGCAGCACCACCGAGCAGACCAAGCTGTACGG
CAGCGGCAACAAGCTGATCACAGTGGGCAGCAGCAACTACCAGCAGAGCTTCGTGCCCAG
CCCTGGCGCCAGACCCCAGGTGAACGGCCAGAGCGGCAGAATCGACTTCCACTGGCTGAT
CCTGAACCCCAACGACACCGTGACCTTCAGCTTCAACGGCGCCTTCATCGCCCCCGACAG
AGCCAGCTTCCTGAGGGGCAAGAGCATGGGCATCCAGAGCGAGGTGCAGGTGGACGCCAA
TTGTGAGGGCGACTGCTACCACTCCGGCGGCACCATCATCAGCAACCTGCCCTTCCAGAA
CATCAACAGCAGGGCTGTGGGCAAGTGCCCTAGATACGTGAAGCAGGAGAGCCTGCTGCT
GGCCACCGGCATGAAGAACGTGCCCGAGATCCCCAAGAGGAGGAGGAGAGGCCTGTTCGG
CGCCATCGCCGGCTTCATCGAGAACGGCTGGGAGGGCCTGATCGACGGCTGGTACGGCTT
CAGACACCAGAACGCCCAGGGCGAGGGCACAGCCGCCGACTACAAGAGCACCCAGAGCGC
CATCGACCAGATCACCGGCAAGCTGAACCGGCTGATCGAGAAAACCAACCAGCAGTTCGA
GCTGATCGACAACGAGTTCACCGAGGTGGAGAGGCAGATCGGCAACGTGATCAACTGGAC
CCGGGACAGCATGACCGAAGTGTGGAGCTACAACGCCGAGCTGCTGGTCGCCATGGAGAA
CCAGCACACCATCGACCTGGCCGACAGCGAGATGAATAAGCTGTACGAGCGGGTGAAGAG
GCAGCTGAGAGAGAACGCCGAGGAGGACGGCACCGGCTGCTTCGAGATCTTCCACAAGTG
TGACGACGACTGTATGGCCTCCATCCGGAACAACACCTACGACCACAGCAAGTACAGGGA
GGAGGCCATCCAGAACCGGATCCAGATCGACCCTGTGAAGCTGTCCAGCGGCTACAAGGA
CGTGATCCTGTGGTTCAGCTTCGGCGCCAGCTGCTTCATCCTGCTGGCCATCGCCATGGG
CCTGGTGTTCATCTGTGTGAAGAACGGCAACATGAGGTGTACCATCTGTATCGGCGGCGA
CTACAAGGATGACGACGACAAGTGATGACCGGTTAACTCGAGCTC

FIGURE 7E

GGTACCGCGGCCGCGCGCCACCATGGAGAAGATCGTGCTGCTGCTGGCTATCGTGTCCCT
GGTGAAGAGCGACCAGATCTGTATCGGCTACCACGCCAACAATAGCACCGAGCAGGTGGA
CACCATCATGGAGAAAAACGTGACCGTGACCCACGCCCAGGATATTCTGGAGAAAACCCA
CAACGGCAAGCTGTGTGACCTGGACGGCGTGAAGCCTCTGATCCTGAGGGATTGTAGCGT
GGCCGGCTGGCTGCTGGGCAACCCCATGTGTGACGAGTTCATCAACGTGCCCGAGTGGAG
CTACATCGTGGAGAAGGCCAACCCCACCAACGATCTGTGCTACCCCGGCAGCTTCAACGA
TTACGAGGAGCTGAAGCACCTGCTGTCCCGGATCAACCACTTCGAGAAGATCCAGATCAT
CCCCAAGAGCAGCTGGAGCGACCACGAGGCCTCCAGCGGCGTGTCCAGCGCCTGCCCCTA
CCTGGGCAGCCCTAGCTTCTTCCGGAACGTGGTGTGGCTGATCAAGAAGAACAGCACCTA
CCCCACCATCAAGAAGAGCTACAACAACACCAACCAGGAGGACCTGCTGGTCCTGTGGGG
CATCCACCACCCCAACGACGCCGCCGAGCAGACCAGACTGTACCAGAACCCCACCACCTA
CATCAGCATCGGCACCTCCACCCTGAATCAGAGGCTGGTGCCCAAGATCGCCACCAGGTC
CAAGGTGAACGGCCAGAGCGGCAGGATGGAGTTCTTCTGGACCATCCTGAAGCCCAACGA
TGCCATCAACTTCGAGAGCAACGGCAACTTCATCGCCCCTGAGTACGCCTACAAGATCGT
GAAGAAGGGCGACAGCGCCATCATGAAGTCCGAGCTGGAGTACGGCAACTGTAACACCAA
GTGCCAGACCCCCATGGGCGCCATCAATAGCAGCATGCCCTTCCACAACATCCACCCCCT
GACCATCGGCGAGTGCCCCAAGTACGTGAAGAGCAACAGGCTGGTGCTGGCCACCGGCCT
GAGAAATAGCCCCCAGCGGGAGAGCAGGAGAAAGAAGAGGGGCCTGTTCGGCGCCATCGC
CGGCTTCATCGAGGGCGGCTGGCAGGGCATGGTGGACGGCTGGTACGGCTACCACCACAG
CAACGAGCAGGGCAGCGGCTACGCCGCCGACAAGGAGAGCACCCAGAAGGCCATCGACGG
CGTCACCAACAAGGTGAACAGCATCATCGACAAGATGAACACCCAGTTCGAGGCTGTGGG
CAGGGAGTTCAACAACCTGGAGCGGCGGATCGAGAACCTGAACAAGAAGATGGAGGACGG
CTTCCTGGATGTGTGGACCTACAACGCCGAGCTGCTGGTGCTGATGGAGAACGAGCGGAC
CCTGGACTTCCACGACAGCAACGTGAAGAACCTGTACGACAAAGTGAGGCTGCAGCTGAG
GGACAATGCCAAGGAGCTGGGCAACGGCTGCTTCGAGTTCTACCACAAGTGTGACAACGA
GTGTATGGAGAGCATCCGGAACGGCACCTACAACTACCCCCAGTACAGCGAGGAGGCCAG
GCTGAAGAGAGAGGAGATCAGCGGGGTCAAGCTGGAGAGCATTGGCACCTACCAGATCCT
GAGCATCTACAGCACCGTGGCCAGCAGCCTGGCCCTGGCCATTATGATGGCCGGCCTGAG
CCTGTGGATGTGTAGCAACGGCAGCCTGCAGTGCAGGATCTGTATT