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*[Continued on next page]*

(54) Title: FUNCTIONAL INFLUENZA VIRUS LIKE PARTICLES (VLPS)

**ATG**AATCCAAATCAAAAGATAATAGCACTTGGCTCTGTTTCTATAACTATTGCGACAATATG
TTTACTCATGCAGATTGCCATCTTAGCAACGACTATGACACTACATTTCAATGAATGTACCA
ACCCATCGAACAATCAAGCAGTGCCATGTGAACCAATCATAATAGAAAGGAACATAACAGAG
ATAGTGCATTTGAATAATACTACCATAGAGAAGGAAAGTTGTCCTAAAGTAGCAGAATACAA
GAATTGGTCAAAACCGCAATGTCAAATTACAGGGTTCGCCCCTTTCTCCAAGGACAACTCAA
TTAGGCTTTCTGCAGGCGGGGATATTTGGGTGACAAGAGAACCTTATGTATCGTGCGGTCTT
GGTAAATGTTACCAATTTGCACTTGGGCAGGGAACCACTTTGAACAACAAACACTCAAATGG
CACAATACATGATAGGAGTCCCCATAGAACCCTTTTAATGAACGAGTTGGGTGTTCCATTTC
ATTTGGGAACCAAACAAGTGTGCATAGCATGGTCCAGCTCAAGCTGCCATGATGGGAAGGCA
TGGTTACATGTTTGTGTCACTGGGGATGATAGAAATGCGACTGCTAGCATCATTTATGATGG
GATGCTTACCGACAGTATTGGTTCATGGTCTAAGAACATCCTCAGAACTCAGGAGTCAGAAT
GCGTTTGCATCAATGGAACTTGTACAGTAGTAATGACTGATGGAAGTGCATCAGGAAGGGCT
GATACTAAAATACTATTCATTAGAGAAGGGAAAATTGTCCACATTGGTCCACTGTCAGGAAG
TGCTCAGCATGTGGAGGAATGCTCCTGTTACCCCCGGTATCCAGAAGTTAGATGTGTTTGCA
GAGACAATTGGAAGGGCTCCAATAGACCCGTGCTATATATAAATGTGGCAGATTATAGTGTT
GATTCTAGTTATGTGTGCTCAGGACTTGTTGGCGACACACCAAGAAATGACGATAGCTCCAG
CAGCAGTAACTGCAGGGATCCTAATAACGAGAGAGGGGGCCCAGGAGTGAAAGGGTGGGCCT
TTGACAATGGAAATGATGTTTGGATGGGACGAACAATCAAGAAAGATTCGCGCTCTGGTTAT
GAGACTTTCAGGGTCGTTGGTGGTTGGACTACGGCTAATTCCAAGTCACAAATAAATAGGCA
AGTCATAGTTGACAGTGATAACTGGTCTGGGTATTCTGGTATATTCTCTGTTGAAGGAAAAA
CCTGCATCAACAGGTGTTTTTATGTGGAGTTGATAAGAGGGAGACCACAGGAGACCAGAGTA
TGGTGGACTTCAAATAGCATCATTGTATTTTGTGGAACTTCAGGTACCTATGGAACAGGCTC
ATGGCCCGATGGAGCGAATATCAATTTCATGTCTATA**TAA**

(57) Abstract: The present invention discloses and claims virus like particles (VLPs) that express and/or contains seasonal influenza virus proteins, avian influenza virus proteins and/or influenza virus proteins from viruses with pandemic potential. The invention includes vector constructs comprising said proteins, cells comprising said constructs, formulations and vaccines comprising VLPs of the inventions. The invention also includes methods of making and administrating VLPs to vertebrates, including methods of inducing substantial immunity to either seasonal and avian influenza, or at least one symptom thereof.

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# Functional Influenza Virus Like Particles (VLPs)

[0001] This Application claims priority to provisional application 60/727,513, filed October 18, 2005, provisional application 60/780,847, filed March 10, 2006, provisional application 60/800,006, filed May 15, 2006, provisional application 60/831,196, filed July 17, 2006, provisional application 60/832,116, filed July 21, 2006, and provisional application 60/845,495, filed September 19, 2006 all of which are incorporated herein by reference in their entireties for all proposes.

## BACKGROUND OF INVENTION

[0002] Influenza virus is a member of Orthomyxoviridae family (for review, see Murphy and Webster, 1996). There are three subtypes of influenza viruses designated A, B, and C. The influenza virion contains a segmented negative-sense RNA genome. The influenza virion includes the following proteins: hemagglutinin (HA), neuraminidase (NA), matrix (M1), proton ion-channel protein (M2), nucleoprotein (NP), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), and nonstructural protein 2 (NS2) proteins. The HA, NA, M1, and M2 are membrane associated, whereas NP, PB1, PB2, PA, and NS2 are nucleocapsid associated proteins. The NS1 is the only nonstructural protein not associated with virion particles but specific for influenza-infected cells. The M1 protein is the most abundant protein in influenza particles. The HA and NA proteins are envelope glycoproteins, responsible for virus attachment and penetration of the viral particles into the cell, and the sources of the major immunodominant epitopes for virus neutralization and protective immunity. Both HA and NA proteins are considered the most important components for prophylactic influenza vaccines.

[0003] Influenza virus infection is initiated by the attachment of the virion surface HA protein to a sialic acid-containing cellular receptor (glycoproteins and glycolipids). The NA protein mediates processing of the sialic acid receptor, and virus penetration into the cell depends on HA-dependent receptor-mediated endocytosis. In the acidic confines of internalized endosomes containing an influenza virion, the HA protein undergoes conformational changes that lead to fusion of viral and host cell membranes followed by virus uncoating and M2-mediated release of M1 proteins from nucleocapsid-associated ribonucleoproteins (RNPs), which migrate into the cell nucleus for viral RNA synthesis.

Antibodies to HA molecule can prevent virus infection by neutralizing virus infectivity, whereas antibodies to NA proteins mediate their effect on the early steps of viral replication.

[0004] Inactivated influenza A and B virus vaccines are licensed currently as trivalent vaccines for parenteral administration. These trivalent vaccines are produced as monovalent bulk in the allantoic cavity of embryonated chick eggs, purified by rate zonal centrifugation or column chromatography, inactivated with formalin or β-propiolactone, and formulated as a blend of the two strains of type A and the type B strain of influenza viruses in circulation among the human population for a given year. The available commercial influenza vaccines are whole virus (WV) or subvirion (SV; split or purified surface antigen) virus vaccines. The WV vaccine contains intact, inactivated virions. SV vaccines treated with solvents such as tri-n-butyl phosphate (Flu-Shield, Wyeth-Lederle) contain nearly all of the viral structural proteins and some of the viral envelopes. SV vaccines solubilized with Triton X-100 (Fluzone, Sanofi-Aventis; Fluvirin, Novartis) contain aggregates of HA monomers, NA, and NP principally, although residual amounts of other viral structural proteins are present. A live attenuated cold-adapted virus vaccine (FluMist, MedImmune) was granted marketing approval recently by the FDA for commercial usage as an intranasally delivered vaccine indicated for active immunization and the prevention of disease caused by influenza A and B viruses in healthy children and adolescents, 5-17 years of age and healthy adults 18-49 years of age.

[0005] Several recombinant products have been developed as recombinant influenza vaccine candidates. These approaches have focused on the expression, production, and purification of influenza virus type A HA and NA proteins, including expression of these proteins using baculovirus infected insect cells (Crawford et al, 1999; Johansson, 1999; Treanor *et al.*, 1996), viral vectors (Pushko *et al.*, 1997; Berglund *et al.*, 1999), and DNA vaccine constructs (Olsen *et al.*, 1997).

[0006] Crawford *et al.* (1999) demonstrated that influenza HA expressed in baculovirus infected insect cells is capable of preventing lethal influenza disease caused by avian H5 and H7 influenza subtypes. At the same time, another group demonstrated that baculovirus-expressed influenza HA and NA proteins induce immune responses in animals superior to those induced by a conventional vaccine (Johansson *et al.*, 1999). Immunogenicity and efficacy of baculovirus-expressed hemagglutinin of equine influenza virus was compared to a homologous DNA vaccine candidate (Olsen *et al.*, 1997). Taken together, the data demonstrated that a high degree of protection against influenza virus challenge can be induced with recombinant HA or NA proteins, using various experimental approaches and in different animal models.

[0007] Lakey *et al.* (1996) showed that a baculovirus-derived influenza HA vaccine was well-tolerated and immunogenic in human volunteers in a Phase I dose escalation safety study. However, results from Phase II studies conducted at several clinical sites in human volunteers vaccinated with several doses of influenza vaccines comprised of HA and/or NA proteins indicated that the recombinant subunit protein vaccines did not elicit protective immunity [G. Smith, Protein Sciences; M. Perdue, USDA, Personal Communications]. These results indicated that conformational epitopes displayed on the surface of HA and NA peplomers of infectious virions were important in the elicitation of neutralizing antibodies and protective immunity.

[0008] Regarding the inclusion of other influenza proteins in recombinant influenza vaccine candidates, a number of studies have been carried out, including the experiments involving influenza nucleoprotein, NP, alone or in combination with M1 protein (Ulmer *et al.*, 1993; Ulmer *et al.*, 1998; Zhou *et al.*, 1995; Tsui *et al.*, 1998). These vaccine candidates, which were composed of quasi-invariant inner virion proteins, elicited a broad spectrum immunity that was primarily cellular (both $CD4^+$ and $CD8^+$ memory T cells). These experiments involved the use of the DNA or viral genetic vectors. Relatively large amounts of injected DNA were needed, as results from experiments with lower doses of DNA indicated little or no protection (Chen *et al.*, 1998). Hence, further preclinical and clinical research may be required to evaluate whether such DNA-based approaches involving influenza NP and M1 are safe, effective, and persistent.

[0009] Recently, in an attempt to develop more effective vaccines for influenza, particulate proteins were used as carriers of influenza M2 protein epitopes. The rationale for development of an M2-based vaccine was that in animal studies protective immunity against influenza was elicited by M2 proteins (Slepushkin *et al.*, 1995). Neirynck *et al.* (1999) used a 23-aa long M2 transmembrane domain as an amino terminal fusion partner with the hepatitis B virus core antigen (HBcAg) to expose the M2 epitope(s) on the surface of HBcAg capsid-like particles. However, in spite of the fact that both full-length M2 protein and M2-HBcAg VLP induced detectable antibodies and protection in mice, it was unlikely that future influenza vaccines would be based exclusively on the M2 protein, as the M2 protein was present at low copy number per virion, was weakly antigenic, was unable to elicit antibodies that bound free influenza virions, and was unable to block virus attachment to cell receptors (*i.e.* virus neutralization).

**[0010]** Since previous research has shown that the surface influenza glycoproteins, HA and NA, are the primary targets for elicitation of protective immunity against influenza virus and that M1 provides a conserved target for cellular immunity to influenza, a new vaccine candidate may include these viral antigens as a protein macromolecular particle, such as virus-like particles (VLPs). Further, the particle with these influenza antigens may display conformational epitopes that elicit neutralizing antibodies to multiple strains of influenza viruses.

**[0011]** Several studies have demonstrated that recombinant influenza proteins could self-assemble into VLPs in cell culture using mammalian expression plasmids or baculovirus vectors (Gomez-Puertas *et al.*, 1999; Neumann *et al.*, 2000; Latham and Galarza, 2001). Gomez-Puertas *et al.* (1999) demonstrated that efficient formation of influenza VLP depends on the expression levels of viral proteins. Neumann *et al.* (2000) established a mammalian expression plasmid-based system for generating infectious influenza virus-like particles entirely from cloned cDNAs. Latham and Galarza (2001) reported the formation of influenza VLPs in insect cells infected with recombinant baculovirus co-expressing HA, NA, M1, and M2 genes. These studies demonstrated that influenza virion proteins may self-assemble upon co-expression in eukaryotic cells.

## SUMMARY OF INVENTION

**[0012]** The present invention provides for a virus like particle (VLP) comprising an influenza virus M1 protein and influenza virus H5 and N1 hemagglutinin and neuraminidase proteins. In one embodiment, the M1 protein is derived from a different influenza virus strain as compared to the H5 and N1 proteins. In another embodiment, said H5 or N1 are from a H5N1 clade 1 influenza virus.

**[0013]** The present invention also provides for a VLP expressed from a eukaryotic cell comprising one or more nucleic acids encoding influenza H5 and N1 proteins and an influenza M1 protein under conditions that permit the formation of VLPs. In one embodiment, said eukaryotic cell is selected from the group consisting of yeast, insect, amphibian, avian and mammalian cells. In other embodiment, said eukaryotic cell is an insect cell.

[0014] The present invention also provides for a VLP that elicits neutralizing antibodies in a human or animal that are protective against influenza infection when administered to said human or animal.

[0015] The present invention also provides for an immunogenic composition comprising an effective dose of a VLP of the invention. In one embodiment, said composition comprises an adjuvant.

[0016] The present invention also provides for a vaccine comprising an effective dose of a VLP of the invention. In one embodiment, said vaccine comprises at least a second VLP which comprises HA and NA from different influenza strains. In another embodiment, said vaccine comprises an adjuvant.

[0017] The present invention also provides for a method of inducing substantial immunity to influenza virus infection in an animal, comprising administering at least one effective dose of a vaccine comprising the VLP of the invention. In one embodiment, said vaccine is administered to an animal orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

[0018] The present invention also provides for the use of a VLP of the invention for the preparation of a vaccine for an animal, wherein the vaccine induces substantial immunity to influenza virus infection in said animal.

[0019] The present invention also provides for a method of making a VLP of the invention, comprising expressing M1, HA and NA proteins in a eukaryotic cell.

[0020] The present invention provides for a vaccine comprising an influenza VLP, wherein said VLP comprises influenza M1, HA and NA proteins, wherein said vaccine induces substantial immunity to influenza virus infection in a human. In one embodiment, said vaccine comprises an influenza VLP, wherein said VLP consists essentially of influenza M1, HA and NA proteins, wherein said vaccine induces substantial immunity to influenza virus infection in a human. In another embodiment, said vaccine comprises an influenza VLP, wherein said VLP comprises influenza proteins selected from the group consisting of influenza M1, HA and NA proteins, wherein said vaccine induces substantial immunity to influenza virus infection in a human.

[0021] The present invention also provides for the use of an influenza VLP, wherein said VLP comprises influenza M1, HA and NA proteins, for the preparation of a vaccine, wherein the vaccine induces substantial immunity to influenza virus infection in a human.

[0022] Thus, the invention provides a macromolecular protein structure containing (a) a first influenza virus M1 protein and (b) an additional structural protein, which may include a second or more influenza virus M1 protein; a first, second or more influenza virus HA protein; a first, second, or more influenza virus NA protein; and a first, second, or more influenza virus M2 protein. If the additional structural protein is not from a second or more influenza virus M1 protein, then both or all members of the group, *e.g.*, first and second influenza M2 virus proteins are included. As such, there is provided a functional influenza protein structure, including a subviral particle, VLP, or capsomer structure, or a portion thereof, a vaccine, a multivalent vaccine, and mixtures thereof consisting essentially of influenza virus structural proteins produced by the method of the invention. In a particularly preferred embodiment, the influenza macromolecular protein structure includes influenza virus HA, NA, and M1 proteins that are the expression products of influenza virus genes cloned as synthetic fragments from a wild type virus.

[0023] The macromolecular protein structure may also include an additional structural protein, for example, a nucleoprotein (NP), membrane proteins from species other than noninfluenza viruses and a membrane protein from a non-influenza source, which are derived from avian or mammalian origins and different subtypes of influenza virus, including subtype A and B influenza viruses. The invention may include a chimeric macromolecular protein structure, which includes a portion of at least one protein having a moiety not produced by influenza virus.

[0024] Prevention of influenza may be accomplished by providing a macromolecular protein structure that may be self-assembled in a host cell from a recombinant construct. The macromolecular protein structure of the invention has the ability to self-assemble into homotypic or heterotypic virus-like particles (VLPs) that display conformational epitopes on HA and NA proteins, which elicit neutralizing antibodies that are protective. The composition may be a vaccine composition, which also contains a carrier or diluent and/or an adjuvant. The functional influenza VLPs elicit neutralizing antibodies against one or more strains or types of influenza virus depending on whether the functional influenza VLPs contain HA and/or NA proteins from one or more viral strains or types. The vaccine may include influenza virus proteins that are wild type influenza virus proteins. Preferably, the structural proteins containing the influenza VLP, or a portion of thereof, may be derived from the various strains of wild type influenza viruses. The influenza vaccines may be administered to humans or animals to elicit protective immunity against one or more strains or types of influenza virus.

**[0025]** The macromolecular protein structures of the invention may exhibit hemagglutinin activity and/or neuraminidase activity.

**[0026]** The invention provides a method for producing a VLP derived from influenza by constructing a recombinant construct that encodes influenza structural genes, including M1, HA, and at least one structural protein derived from influenza virus. A recombinant construct is used to transfect, infect, or transform a suitable host cell with the recombinant baculovirus. The host cell is cultured under conditions which permit the expression of M1, HA and at least one structural protein derived from influenza virus and the VLP is formed in the host cell. The infected cell media containing a functional influenza VLP is harvested and the VLP is purified. The invention also features an additional step of co-transfecting, co-infecting or co-transforming the host cell with a second recombinant construct which encodes a second influenza protein, thereby incorporating the second influenza protein within the VLP. Such structural proteins may be derived from influenza virus, including NA, M2, and NP, and at least one structural protein is derived from avian or mammalian origins. The structural protein may be a subtype A and B influenza viruses. According to the invention, the host cell may be a eukaryotic cell. In addition, the VLP may be a chimeric VLP.

**[0027]** The invention also features a method of formulating a drug substance containing an influenza VLP by introducing recombinant constructs encoding influenza viral genes into host cells and allowing self-assembly of the recombinant influenza viral proteins into a functional homotypic or heterotypic VLP in cells. The influenza VLP is isolated and purified and a drug substance is formulated containing the influenza VLP. The drug substance may further include an adjuvant. In addition, the invention provides a method for formulating a drug product, by mixing such a drug substance containing an influenza VLP with a lipid vesicle, *i.e.*, a non-ionic lipid vesicle. Thus, functional homotypic or heterotypic VLPs may bud as enveloped particles from the infected cells. The budded influenza VLPs may be isolated and purified by ultracentrifugation or column chromatography as drug substances and formulated alone or with adjuvants such as Novasomes®, a product of Novavax, Inc., as drug products such as vaccines. Novasomes®, which provide an enhanced immunological effect, are further described in U.S. Pat. No. 4,911,928, which is incorporated herein by reference.

[0028] The invention provides a method for detecting humoral immunity to influenza virus infection in a vertebrate by providing a test reagent including an effective antibody-detecting amount of influenza virus protein having at least one conformational epitope of an influenza virus macromolecular structure. The test reagent is contacted with a sample of bodily fluid from a vertebrate to be examined for influenza virus infection. Influenza virus specific antibodies contained in the sample are allowed to bind to the conformational epitope of an influenza virus macromolecular structure to form antigen-antibody complexes. The complexes are separated from unbound complexes and contacted with a detectably labeled immunoglobulin-binding agent. The amount of the detectably labeled immunoglobulin-binding agent that is bound to the complexes is determined.

[0029] Influenza virus may be detected in a specimen from an animal or human suspected of being infected with the virus by providing antibodies, which have a detectable signal producing label, or are attached to a detectably labeled reagent, having specificity to at least one conformational epitope of the particle of the influenza virus. The specimen is contacted with antibodies and the antibodies are allowed to bind to the influenza virus. The presence of influenza virus in the specimen is determined by means of the detectable label.

[0030] The invention provides methods for treatment, prevention, and generating a protective immune response by administering to a vertebrate an effective amount of the composition of the invention.

[0031] Alternatively, the influenza VLP drug substance may be formulated as laboratory reagents used for influenza virus structure studies and clinical diagnostic assays. The invention also provides a kit for treating influenza virus by administering an effective amount of a composition of the invention and directions for use.

[0032] The invention also provides for a VLP comprising HA, NA and M1 proteins derived from an avian influenza virus which can cause morbidity or mortality in a vertebrate. In one embodiment, said HA, NA and M1 proteins are derived from an avian influenza type A virus. In another embodiment the HA is selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16 and the NA is selected from the group consisting of N1, N2, N3, N4, N5, N6, N7, N8 and N9. In a further embodiment, said HA and NA proteins are H5 and N1, respectively. In another embodiment, said HA and NA proteins are H9 and N2, respectively. In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In one embodiment, the VLP consists essentially of HA, NA and M1 proteins, i.e., these are substantially the only influenza proteins in the VLP.

**[0033]** The invention also provides for a method of producing a VLP, comprising transfecting vectors encoding avian influenza virus proteins into a suitable host cell and expressing said avian influenza virus proteins under condition that allow VLPs to be formed. In one embodiment, this method involves transfecting a host cell with recombinant DNA molecules that encode only the HA, NA and M1 influenza proteins.

**[0034]** The invention also comprises an antigenic formulation comprising a VLP comprising HA, NA and M1 proteins derived from an avian influenza virus which can cause morbidity or mortality in a vertebrate. In another embodiment, the HA is selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16 and the NA is selected from the group consisting of N1, N2, N3, N4, N5, N6, N7, N8 and N9. In a further embodiment, said HA and NA proteins are H5 and N1, respectively. In another embodiment, said HA and NA proteins are H9 and N2, respectively. In a further embodiment, said antigenic formulation is administered to the subject orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

**[0035]** The invention further provides for a method of vaccinating a vertebrate against avian influenza virus comprising administering to said vertebrate a protection-inducing amount of a VLP comprising HA, NA and M1 proteins derived from an avian influenza virus.

**[0036]** This invention also comprises a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an influenza VLP. In one embodiment, said VLP consists essentially of HA, NA and M1. In another embodiment, said VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1. In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully.

**[0037]** This invention also comprises a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an avian influenza VLP. In one embodiment, said influenza VLP consists essentially of avian HA, NA and M1. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of avian HA, NA and M1.

[0038] This invention further comprises a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a seasonal influenza VLP. In one embodiment, said influenza VLP consists essentially of seasonal HA, NA and M1. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of seasonal HA, NA and M1.

[0039] This invention further comprises a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of at least one seasonal influenza VLP. In one embodiment, said influenza VLP comprises seasonal influenza HA, NA and M1. In another embodiment, said influenza VLP consists essentially of seasonal influenza HA, NA and M1.

[0040] This invention further comprises a method of inducing a substantially protective antibody response to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an influenza VLP.

[0041] This invention comprises a method of inducing a substantially protective cellular immune response to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an influenza VLP.

[0042] This invention further comprises a method of formulating a vaccine that induces substantial immunity to influenza virus infection or at least one symptom thereof to a subject, comprising adding to said formulation an effective dose of an influenza VLP. In one embodiment, said substantial immunity to influenza virus infection or at least one symptom thereof is delivered in one dose. In another embodiment, said substantial immunity to influenza virus infection or at least one symptom thereof is delivered in multiple doses.

[0043] This invention further comprises a vaccine comprising an influenza VLP, wherein said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof when administered to a subject. In one embodiment, said influenza VLP is an avian influenza VLP. In another embodiment, said influenza VLP is a seasonal influenza VLP.

[0044] This invention further comprises an antigenic formulation comprising an influenza VLP, wherein said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof when administered to a subject. In one embodiment, said influenza VLP is an avian influenza VLP. In another embodiment, said influenza VLP is a seasonal influenza VLP.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0045] FIG. 1 depicts the nucleotide sequence of avian influenza A/Hong Kong/1073/99 (H9N2) virus neuraminidase (NA) gene (SEQ ID NO:1).

[0046] FIG. 2 depicts the nucleotide sequence of avian influenza A/Hong Kong/1073/99 (H9N2) virus hemagglutinin (HA) gene (SEQ ID NO:2).

[0047] FIG. 3 depicts the nucleotide sequence of avian influenza A/Hong Kong/1073/99 (H9N2) virus matrix protein M1 (M1) gene (SEQ ID NO:3).

[0048] FIG. 4 depicts the transfer vectors for construction of recombinant baculoviruses for expression of avian influenza A/Hong Kong/1073/99 (H9N2) HA, NA, and M1 proteins. FIG. 4A depicts a transfer vector for expression of individual genes and FIG. 4B depicts the transfer vector for multi-expression of the genes.

[0049] FIG. 5 depicts the expression of avian influenza A/Hong Kong/1073/99 (H9N2) virus HA, NA, and M1 proteins in Sf-9S cells.

[0050] FIG. 6 depicts the purification of avian influenza A/Hong Kong/1073/99 (H9N2) VLPs by the sucrose density gradient method.

[0051] FIG. 7 depicts the detection of influenza virus protein by gel filtration chromatography. The antibodies used in the Western blot analyses are as follows: (A) rabbit anti-H9N2; (b) murine anti-M1 mAb; and (C) murine anti-BACgp64.

[0052] FIG. 8 depicts the detection of avian influenza A/Hong Kong/1073/99 (H9N2) proteins including subviral particles, VLP, and VLP complexes, by electron microscopy.

[0053] FIG. 9 depicts the hemagglutination activity of purified avian influenza A/Hong Kong/1073/99 (H9N2) VLPs.

[0054] FIG. 10 depicts the neuraminidase activity of purified avian influenza A/Hong Kong/1073/99 (H9N2) VLPs.

[0055] FIG. 11 depicts the immunization and bleed schedule for the immunogenicity study of recombinant influenza with purified avian influenza A/Hong Kong/1073/99 (H9N2) VLPs in mice.

[0056] FIG. 12 depicts the results of an immunogenicity study in mice immunized with recombinant influenza H9N2 VLPs. FIG. 12A depicts sera from BALB/c mice immunized with recombinant VLPs comprised of HA, NA, and M1 proteins from avian influenza virus type A/H9N2/Hong Kong/1073/99. FIG. 12B depicts sera from New Zealand white rabbits immunized with inactivated avian influenza virus type A H9N2 were reacted with Western blots containing inactivated avian influenza virus type A H9N2 (lanes 1 and 3) or cold-adapted avian influenza virus type A H9N2 (lanes 2 and 4).

[0057] FIG. 13 depicts the geometric mean antibody responses in BALB/c mice after a primary and secondary immunization.

[0058] FIG. 14 depicts serum hemagglutinin inhibition (HI) responses in BALB/c mice.

[0059] FIG. 15 depicts weight loss (%) in BALB/c mice challenged with H9N2 influenza.

[0060] FIG. 16 depicts lung virus titers at 3 and 5 days post challenge with H9N2.

[0061] FIGS. 17A, 17B and 17C depict mice antibody response to A/Fujian/411/2002 when immunized with H3N2 VLP.

[0062] FIG. 18 A and B depict mice IgG antibody isotypes

[0063] FIG. 19 hemagglutinin inhibition (HI) antibody responses in SD Rats immunized with H9N2 VLP vaccine.

[0064] FIGS. 20A and 20B depict hemagglutinin inhibition (HI) antibody responses to different doses of H9N2 VLPs with and without adjuvant in BALB/c mice.

[0065] FIG 21 depicts serum hemagglutinin inhibition (HI) responses in BALB/c mice between different doses of VLPs.

[0066] FIG 22 depicts serum hemagglutinin inhibition (HI) responses in ferrets.

[0067] FIG 23 depicts serum hemagglutinin inhibition (HI) responses from serum pulled on days 21 and 42 from ferrets after administration of different strains of H3N2 VLPs.

[0068] FIG 24 depicts anti-HA Antibody (Endpoint Dilution Titer) of mice inoculated intramuscularly with H5N1 (Vietnam/1203/2003) VLPs at low doses.

[0069] FIG 25 depicts anti-HA Antibody (Endpoint Dilution Titer) of mice inoculated intranasally with H5N1 (Vietnam/1203/2003) VLPs at low doses.

[0070] FIG 26 depicts an example for manufacturing, isolating and purifying VLPs of the invention.

[0071] FIG 27 depicts mice inoculated with H3N2 VLPs given intramuscularly and subsequently challenged intranasally with A/Aichi/2/68x31 (H3N2) virus.

[0072] FIG 28 depicts mice inoculated with H3N2 VLPs given intranasally and subsequently challenged intranasally with A/Aichi/2/68x31 (H3N2) virus.

[0073] FIG 29 depicts virus shedding in nasal washes of ferret inoculated with H9N2 VLP vaccine and subsequently challenged intranasally with H9N2 virus.

[0074] FIG 30A, 30B, 30C, 30D, 30E, 30F, 30G, 30H depicts hemagglutinin inhibition (HI) antibody responses in mice after inoculation with different doses of A/Fujian/411/2002

(H3N2) VLPs intramuscularly or intranasally tested against different H3N2 strains of influenza viruses.

## DETAILED DESCRIPTION OF THE INVENTION

**[0075]** As used herein, the term "baculovius," also known as baculoviridae, refers to a family of enveloped DNA viruses of arthropods, members of which may be used as expression vectors for producing recombinant proteins in insert cell cultures. The virion contains one or more rod-shaped nucleocapsids containing a molecule of circular supercoiled double-stranded DNA (Mr $54 \times 10^6$-$154 \times 10^6$). The virus used as a vector is generally *Autographa californica* nuclear polyhedrosis virus (NVP). Expression of introduced genes is under the control of the strong promoter that normally regulates expression of the polyhedron protein component of the large nuclear inclusion in which the viruses are embedded in the infected cells.

**[0076]** As used herein, the term "derived from" refers to the origin or source, and may include naturally occurring, recombinant, unpurified, or purified molecules. The proteins and molecules of the present invention may be derived from influenza or non-influenza molecules.

**[0077]** As used herein the term "first" influenza virus protein, *i.e.*, a first influenza virus M1 protein, refers to a protein, such as M1, HA, NA, and M2, that is derived from a particular strain of influenza virus. The strain or type of the first influenza virus differs from the strain or type of the second influenza virus protein. Thus, "second" influenza virus protein, *i.e.*, the second influenza virus M1 protein, refers to a protein, such as M1, HA, NA, and M2, that is derived from a second strain of influenza virus, which is a different strain or type than the first influenza virus protein.

**[0078]** As used herein, the term "hemagglutinin activity" refers to the ability of HA-containing proteins, VLPs, or portions thereof to bind and agglutinate red blood cells (erythrocytes).

**[0079]** As used herein, the term "neuraminidase activity" refers to the enzymatic activity of NA-containing proteins, VLPs, or portions thereof to cleave sialic acid residues from substrates including proteins such as fetuin.

**[0080]** As used herein, the term "heterotypic" refers to one or more different types or strains of virus.

**[0081]** As used herein, the term "homotypic" refers to one type or strain of virus.

[0082] As used herein, the term "macromolecular protein structure" refers to the construction or arrangement of one or more proteins.

[0083] As used herein, the term "multivalent" vaccine refers to a vaccine against multiple types or strains of influenza virus.

[0084] As used herein, the term "non-influenza" refers to a protein or molecule that is not derived from influenza virus.

[0085] As used herein, the term "vaccine" refers to a preparation of dead or weakened pathogens, or of derived antigenic determinants, that is used to induce formation of antibodies or immunity against the pathogen. A vaccine is given to provide immunity to the disease, for example, influenza, which is caused by influenza viruses. The present invention provides vaccine compositions that are immunogenic and provide protection. In addition, the term "vaccine" also refers to a suspension or solution of an immunogen (*e.g.* VLP) that is administered to a vertebrate to produce protective immunity, i.e., immunity that reduces the severity of disease associated with infection.

[0086] As used herein the term "substantial immunity" refers to an immune response in which when VLPs of the invention are administered to a vertebrate there is an induction of the immune system in said vertebrate which results in the prevention of influenza infection, amelioration of influenza infection or reduction of at least one symptom related to influenza virus infection in said vertebrate. Substantial immunity may also refer to a haemagglutination inhibition (HI) titer of ≥ 40 in a mammal wherein the VLPs of the invention have been administered and have induced an immune response.

[0087] As used herein the term "adjuvant" refers to a compound that, when used in combination with a specific immunogen (*e.g.* a VLP) in a formulation, augments or otherwise alters or modifies the resultant immune response. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen-specific immune responses.

[0088] As used herein the term "immune stimulator" refers to a compound that enhances an immune response *via* the body's own chemical messengers (cytokines). These molecules comprise various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immune stimulator molecules can be administered in the same formulation as the influenza VLPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

**[0089]** As used herein an "effective dose" generally refers to that amount of the VLP of the invention sufficient to induce immunity, to prevent and/or ameliorate influenza virus infection or to reduce at least one symptom of influenza infection and/or to enhance the efficacy of another dose of a VLP. An effective dose may refer to the amount of the VLP sufficient to delay or minimize the onset of an influenza infection. An effective dose may also refer to the amount of the VLP that provides a therapeutic benefit in the treatment or management of influenza infection. Further, an effective dose is the amount with respect to the VLPs of the invention alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of an influenza viral infection. An effective dose may also be the amount sufficient to enhance a subject's (*e.g.*, a human's) own immune response against a subsequent exposure to influenza virus. Levels of immunity can be monitored, *e.g.*, by measuring amounts of neutralizing secretory and/or serum antibodies, *e.g.*, by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay. In the case of a vaccine, an "effective dose" is one that prevents disease or reduces the severity of symptoms.

**[0090]** As used herein the term "avian influenza virus" refers to influenza viruses found chiefly in birds but that can also infect humans or other animals. In some instances, avian influenza viruses may be transmitted or spread from one human to another. An avian influenza virus that infects humans has the potential to cause an influenza pandemic, i.e., morbidity and/or mortality in humans. A pandemic occurs when a new strain of influenza virus (a virus in which human have no natural immunity) emerges, spreading beyond individual localities, possibly around the globe, and infecting many humans at once.

**[0091]** As used herein the term "seasonal influenza virus" refers to the influenza viral strains that have been determined to be passing within the human population for a given influenza season based on epidemiological surveys conducted by National Influenza Centers worldwide. These epidemiological studies, and some isolated influenza viruses, are sent to one of four World Health Organization (WHO) reference laboratories, one of which is at the Centers for Disease Control and Prevention (CDC) in Atlanta for detailed testing. These laboratories test how well antibodies made to the current vaccine react to the circulating virus and new flu viruses. This information, along with information about flu activity, is summarized and presented to an advisory committee of the U.S. Food and Drug Administration (FDA) and at a WHO meeting. These meetings result in the selection of three viruses (two subtypes of influenza A viruses and one influenza B virus) to go into flu vaccines for the following fall and winter. The selection occurs in February for the northern hemisphere and in September for the southern hemisphere. Usually, one or two of the three virus strains in the vaccine changes each year.

**[0092]** As used herein the term “substantially protective antibody response” refers to an immune response mediated by antibodies against an influenza virus, which is exhibited by a vertebrate (*e.g.*, a human), that prevents or ameliorates influenza infection or reduces at least one symptom thereof. VLPs of the invention can stimulate the production of antibodies that, for example, neutralizing antibodies that block influenza viruses from entering cells, blocks replication of said influenza virus by binding to the virus, and/or protect host cells from infection and destruction.

**[0093]** As used herein the term “substantially protective cellular response” refers to an immune response that is mediated by T-lymphocytes and/or other white blood cells against influenza virus, exhibited by a vertebrate (*e.g.*, a human), that prevents or ameliorates influenza infection or reduces at least one symptom thereof. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells (“CTL”s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A “cellular immune response” also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells.

**[0094]** As used herein the term “substantial immunity in a population-wide basis” refers to immunity as a result of VLPs of the invention administered to individuals in a population. The immunity in said individual in said population results in the prevention, amelioration of influenza infection, or reduction of at least one symptom related to influenza virus infection in said individual, and prevents the spread of said influenza virus to others in the population.

The term population is defined as group of individuals (*e.g.* schoolchildren, elderly, healthy individuals etc.) and may comprise a geographic area (*e.g.* specific cities, schools, neighborhoods, workplace, country, state, etc.).

**[0095]** As use herein, the term "antigenic formulation" or "antigenic composition" refers to a preparation which, when administered to a vertebrate, especially a bird or a mammal, will induce an immune response.

**[0096]** As use herein, the term "vertebrate" or "subject" or "patient" refers to any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species. Farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like are also non-limiting examples . The terms "mammals" and "animals" are included in this definition. Both adult and newborn individuals are intended to be covered.

**[0097]** Influenza remains a pervasive public health concern despite the availability of specific inactivated virus vaccines that are 60-80% effective under optimal conditions. When these vaccines are effective, illness is usually averted by preventing viral infection. Vaccine failure can occur as a result of accumulated antigenic differences (antigenic shift and antigenic drift). For example, avian influenza virus type A H9N2 co-circulated with human influenza virus type A Sydney/97 (H3N2) in pigs and led to genetic reassortment and emergence of new strains of human influenza virus with pandemic potential (Peiris *et al.*, 2001). In the event of such antigenic shift, it is unlikely that current vaccines would provide adequate protection.

**[0098]** Another reason for the paucity of influenza vaccine programs is the relatively short persistence of immunity elicited by the current vaccines. Further inadequacy of influenza control measures reflects restricted use of current vaccines because of vaccine reactogenicity and side effects in young children, elderly, and people with allergies to components of eggs, which are used in manufacturing of commercially licensed inactivated virus influenza vaccines.

**[0099]** Additionally, inactivated influenza virus vaccines often lack or contain altered HA and NA conformational epitopes, which elicit neutralizing antibodies and play a major role in protection against disease. Thus, inactivated viral vaccines, as well as some recombinant monomeric influenza subunit protein vaccines, deliver inadequate protection. On the other hand, macromolecular protein structures, such as capsomers, subviral particles, and/or VLPs, include multiple copies of native proteins exhibiting conformational epitopes, which are advantageous for optimal vaccine immunogenicity.

**[00100]** The present invention describes the cloning of avian influenza A/Hong Kong/1073/99 (H9N2) virus HA, NA, and M1 genes into a single baculovirus expression vector alone or in tandem and production of influenza vaccine candidates or reagents comprised of recombinant influenza structural proteins that self-assemble into functional and immunogenic homotypic macromolecular protein structures, including subviral influenza particles and influenza VLP, in baculovirus-infected insect cells.

**[00101]** The present invention describes the cloning of human influenza A/Sydney/5/97 and A/Fujian/411/2002 (H3N2) virus HA, NA, M1, M2, and NP genes into baculovirus expression vectors and production influenza vaccine candidates or reagents comprised of influenza structural proteins that self-assemble into functional and immunogenic homotypic macromolecular protein structures, including subviral influenza particles and influenza VLP, in baculovirus-infected insect cells.

**[00102]** In addition, the instant invention describes the cloning of the HA gene of human influenza A/Sydney/5/97 and A/Fujian/411/2002 (H3N2) virus and the HA, NA, and M1 genes of avian influenza A/Hong Kong/1073/99 (H9N2) into a single baculovirus expression vector in tandem and production influenza vaccine candidates or reagents comprised of influenza structural proteins that self-assemble into functional and immunogenic heterotypic macromolecular protein structures, including subviral influenza particles and influenza VLP, in baculovirus-infected insect cells.

**VLPs of the Invention**

**[00103]** Influenza VLPs of the invention are useful for preparing vaccines against influenza viruses. One important feature of this system is the ability to replace the surface glycoproteins with different subtypes of HA and/or NA or other viral proteins, thus, allowing updating of new influenza antigenic variants every year or to prepare for an influenza pandemic. As antigenic variants of these glycoproteins are identified, the VLPs can be updated to include these new variants (*e.g.* for seasonal influenza vaccines). In addition, surface glycoproteins from potentially pandemic viruses, such as H5N1, or other HA, NA combinations with pandemic potential could be incorporated into VLPs without concern of releasing genes that had not circulated in humans for several decades. This is because the VLPs are not infectious, do not replicate and cannot cause disease. Thus, this system allows for creating a new candidate influenza vaccine every year and/or an influenza pandemic vaccine whenever it is necessary.

**[00104]** There are 16 different hemagglutinin (HA) and 9 different neuraminidase (NA) all of which have been found among wild birds. Wild birds are the primary natural reservoir for all types of influenza A viruses and are thought to be the source of all types of influenza A viruses in all other vertebrates. These subtypes differ because of changes in the hemagglutinin (HA) and neuraminidase (NA) on their surface. Many different combinations of HA and NA proteins are possible. Each combination represents a different type of influenza A virus. In addition, each type can be further classified into strains based on different mutations found in each of its 8 genes.

**[00105]** All known types of influenza A viruses can be found in birds. Usually avian influenza viruses do not infect humans. However, some avian influenza viruses develop genetic variations associated with the capability of crossing the species barrier. Such a virus is capable of causing a pandemic because humans have no natural immunity to the virus and can easily spread from person to person. In 1997, avian influenza virus jumped from a bird to a human in Hong Kong during an outbreak of bird flu in poultry. This virus was identified as influenza virus H5N1. The virus caused severe respiratory illness in 18 people, six of whom died. Since that time, many more cases of known H5N1 infections have occurred among humans worldwide; approximately half of those people have died.

**[00106]** Thus, the present invention encompasses the cloning of HA, NA and M1 nucleotides from avian influenza viruses, influenza viruses with pandemic potential and/or seasonal influenza viruses into expression vectors. The present invention also describes the production of influenza vaccine candidates or reagents comprised of influenza proteins that self-assemble into functional VLPs. All combinations of viral proteins must be co-expressed with a M1 nucleotide.

**[00107]** VLPs of the invention consist or comprise influenza HA, NA and M1 proteins. In one embodiment, said VLP comprises a HA from an avian, pandemic and/or seasonal influenza virus and a NA from an avian, pandemic and/or seasonal influenza virus, wherein said HA is selected from the group consisting of H1, H2, H3, H4, H5, H6, H,7 H8, H9, H10, H11, H12, H13, H14 , H15 and H16 and said NA is selected from the group consisting of N1, N2, N3, N4, N5, N6, N7, N8 and N9. In another embodiment, the invention comprises a

VLP that consists essentially of HA, NA and M1. Said HA and NA can be from the above list of HA and NA. These VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, the HA and/or the NA may exhibit hemagglutinin activity and/or neuraminidase activity, respectively, when expressed on the surface of VLPs.

**[00108]** In another embodiment, said VLP comprises HA and NA of the H5N1 virus and a M1 protein (the M1 protein may or may not be from the same viral strain). In another embodiment, said VLP consists essentially of HA, NA of the H5N1 virus and a M1 protein. These VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In a further embodiment, said VLP consists of HA, NA of the H5N1 virus and a M1 protein. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of H5, N1 and M1 proteins. These VLPs contain H5, N9 and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, H5 and/or N1). In another embodiment, the H5 and/or the N1 may exhibit hemagglutinin activity and/or neuraminidase activity, respectively, when expressed on the surface of VLPs.

**[00109]** In another embodiment, said VLP comprises the HA and NA of the H9N2 virus, and a M1 protein. In another embodiment, said VLP consists essentially of the HA and NA of the H9N2 virus, and a M1 protein. These VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, said VLP consists of the HA and NA of the H9N2 virus, and a M1 protein. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of H9, N2 and M1 proteins. These VLPs contain H9, N2 and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, H9 and/or N2). In another embodiment, the H9 and/or the N2 may exhibit hemagglutinin activity and/or neuraminidase activity, respectively, when expressed on the surface of VLPs.

[00110] In another embodiment, said VLP comprises the HA and NA from an influenza B virus, and a M1 protein. Influenza B viruses are usually found only in humans. Unlike influenza A viruses, these viruses are not classified according to subtype. Influenza B viruses can cause morbidity and mortality among humans, but in general are associated with less severe epidemics than influenza A viruses. In another embodiment, said VLP consists essentially of the HA and NA of the influenza B virus, and a M1 protein. These VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, said VLP consists of the HA and NA of the influenza B virus, and a M1 protein. In another embodiment, the HA and/or the NA may exhibit hemagglutinin activity and/or neuraminidase activity, respectively, when expressed on the surface of VLPs.

[00111] The invention also encompasses variants of the said influenza proteins expressed on or in the VLPs of the invention. The variants may contain alterations in the amino acid sequences of the constituent proteins. The term "variant" with respect to a polypeptide refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. Alternatively, a variant can have "nonconservative" changes, *e.g.*, replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software.

[00112] Natural variants can occur due to antigenic drifts. Antigenic drifts are small changes in the viral proteins that happen continually over time. Thus, a person infected with a particular flu virus strain develops antibody against that virus, as newer virus strains appear, the antibodies against the older strains no longer recognize the newer virus and reinfection can occur. This is why there is a new vaccine for influenza each season. In addition, some changes in an influenza virus can cause influenza virus to cross species. For example, some avian influenza viruses developed genetic variations associated with the capability of

crossing the species barrier. Such a virus is capable of causing a pandemic because people have no natural immunity to the virus and the virus can easily spread from person to person. These naturally occurring variations of the influenza proteins are an embodiment of the invention.

[00113] General texts which describe molecular biological techniques, which are applicable to the present invention, such as cloning, mutation, cell culture and the like, include Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif. (Berger); Sambrook *et al.*, Molecular Cloning--A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 ("Sambrook") and Current Protocols in Molecular Biology, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., ("Ausubel"). These texts describe mutagenesis, the use of vectors, promoters and many other relevant topics related to, *e.g.*, the cloning and mutation of HA and/or NA molecules, etc. Thus, the invention also encompasses using known methods of protein engineering and recombinant DNA technology to improve or alter the characteristics of the influenza proteins expressed on or in the VLPs of the invention. Various types of mutagenesis can be used to produce and/or isolate variant HA, NA and/or M1 molecules and/or to further modify/mutate the polypeptides of the invention. They include but are not limited to site-directed, random point mutagenesis, homologous recombination (DNA shuffling), mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, *e.g.*, involving chimeric constructs, is also included in the present invention. In one embodiment, mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, *e.g.*, sequence, sequence comparisons, physical properties, crystal structure or the like.

[00114] The invention further comprises influenza protein variants which show substantial biological activity, *e.g.*, able to elicit an effective antibody response when expressed on or in a VLP. Such variants include deletions, insertions, inversions, repeats, and substitutions selected according to general rules known in the art so as have little effect on activity.

[00115] Methods of cloning said influenza proteins are known in the art. For example, the influenza gene encoding a specific influenza protein can be isolated by RT-PCR from polyadenylated mRNA extracted from cells which had been infected with an influenza virus. The resulting product gene can be cloned as a DNA insert into a vector. The term "vector" refers to the means by which a nucleic acid can be propagated and/or transferred between organisms, cells, or cellular components. Vectors include plasmids, viruses, bacteriophages, pro-viruses, phagemids, transposons, artificial chromosomes, and the like, that replicate autonomously or can integrate into a chromosome of a host cell. A vector can also be a naked RNA polynucleotide, a naked DNA polynucleotide, a polynucleotide composed of both DNA and RNA within the same strand, a poly-lysine-conjugated DNA or RNA, a peptide-conjugated DNA or RNA, a liposome-conjugated DNA, or the like, that is not autonomously replicating. In many, but not all, common embodiments, the vectors of the present invention are plasmids or bacmids.

[00116] Thus, the invention comprises nucleotides which encode the HA, NA and/or M1 influenza proteins cloned into an expression vector which can be expressed in a cell which induces the formation of VLPs. An "expression vector" is a vector, such as a plasmid that is capable of promoting expression, as well as replication of a nucleic acid incorporated therein. Typically, the nucleic acid to be expressed is "operably linked" to a promoter and/or enhancer, and is subject to transcription regulatory control by the promoter and/or enhancer. In one embodiment, said nucleotides that encode for HA from an avian, pandemic and/or seasonal influenza virus is selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In another embodiment, said nucleotides that encode for NA from an avian, pandemic and/or seasonal influenza virus, is selected from the group consisting of N1, N2, N3, N4, N5, N6, N7, N8 and N9. In another embodiment, said vector comprises of nucleotides that encode the HA, NA and/or M1 influenza protein. In another embodiment, said vector consists of nucleotides that encodes the HA, NA and M1 influenza protein. A preferred expression vector is a baculovirus vector. After the nucleotides encoding said influenza proteins have been cloned said nucleotides can be further manipulated. For example, a person with skill in the art can mutate specific bases in the coding region to produce variants. The variants may contain alterations in the coding regions, non-coding regions, or both. Such variants may increase the immunogenticity of an influenza protein or remove a splice site from a protein or RNA. For example, in one embodiment, the donor and acceptor splicing sites on the influenza M protein (full length) are

mutated to prevent splicing of the M mRNA into M1 and M2 transcripts. In another embodiment the HA is engineered to remove or mutate the cleavage site. For example, wild type H5 HA has a cleavage site that contains multiple basic amino acids (RRRKR). This wild type sequence makes the HA more susceptible to multiple ubiquitous proteases that may be present in host or system expression these HAs. In one embodiment, removing these amino acids can reduce the susceptibility of the HA to various proteases. In another embodiment, the cleavage site can be mutated to remove the cleavage site (*e.g.* mutate to RESR).

**[00117]** The invention also utilizes nucleic acid and polypeptides which encode NA, HA and M1. In one embodiment, an influenza NA nucleic acid or protein is at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NOs 1, 11, 31, 32, 39, 38, 46, 47, 54 or 55. In another embodiment, an influenza HA nucleic acid or protein is at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NOs 2, 10, 56, 57, 58, 27, 28, 29, 30, 37, 36, 33, 34, 35, 42, 43, 44, 45, 50, 51, 52, or 53. In another embodiment, an influenza M1 nucleic acid or protein is at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NOs 12, 40, 41, 48 or 49.

**[00118]** In some embodiments, mutations containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made. Nucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in the human mRNA to those preferred by insect cells such as Sf9 cells). See U.S. patent publication 2005/0118191, herein incorporated by reference in its entirety for all purposes. Examples of optimized codon sequences of the invention are disclosed below (e.g. SEQ ID 42, 44, 46, 48, 50, 52, and 54).

**[00119]** In addition, the nucleotides can be sequenced to ensure that the correct coding regions were cloned and do not contain any unwanted mutations. The nucleotides can be subcloned into an expression vector (*e.g.* baculovirus) for expression in any cell. The above is only one example of how the influenza viral proteins can be cloned. A person with skill in the art understands that additional methods are available and are possible.

**[00120]** The invention also provides for constructs and/or vectors that comprise avian, pandemic and/or seasonal nucleotides which encode for influenza virus structural genes, including NA, M1 and/or HA. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. The constructs and/or vectors that encodes avian, pandemic and/or seasonal influenza virus structural genes, including NA, M1 and/or HA should be operatively linked to an appropriate promoter, such as the AcMNPV polyhedrin promoter (or other baculovirus), phage lambda PL promoter, the *E. coli* lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

**[00121]** The expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (*e.g.*, vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (*e.g.*, canine adenovirus), herpesvirus, and retrovirus. Other vectors that can be used with the invention comprise vectors for use in bacteria, which comprise pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBac1 pWINEO, pSV2CAT, pOG44, pXT1 and pSG, pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan. In one embodiment, said vector that comprises nucleotides encoding for avian, pandemic and/or seasonal influenza virus structural genes, including HA, M1 and/or NA, is pFastBac. In another embodiment, said vector that comprises an insert that consists of nucleotides encoding for avian, pandemic and/or seasonal influenza virus structural genes, comprises HA, M1 and NA, is pFastBac.

**[00122]** Next, the recombinant vector can be transfected, infected, or transformed into a suitable host cell. Thus, the invention provides for host cells which comprise a vector (or vectors) that contain nucleic acids which code for HA, M1 and/or NA and permit the expression of HA, M1 and/or NA in said host cell under conditions which allow the formation of VLPs.

**[00123]** In one embodiment, the recombinant constructs mentioned above could be used to transfect, infect, or transform and can express HA, NA and M1 influenza proteins in eukaryotic cells and/or prokaryotic cells. Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Non limiting examples of insect cells are, *Spodoptera frugiperda* (Sf) cells, *e.g.* Sf9, Sf21, *Trichoplusia ni* cells, *e.g.* High Five cells, and *Drosophila* S2 cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of Candida including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (S. pombe), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are COS cells, baby hamster kidney cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells, CV1 cells, HeLa cells, MDCK cells, Vero and Hep-2 cells. Xenopus laevis oocytes, or other cells of amphibian origin, may also be used. Prokaryotic host cells include bacterial cells, for example, *E. coli*, *B. subtilis*, and mycobacteria.

**[00124]** Vectors, *e.g.*, vectors comprising HA, NA and/or M1 polynucleotides, can be transfected into host cells according to methods well known in the art. For example, introducing nucleic acids into eukaryotic cells can be by calcium phosphate co-precipitation, electroporation, microinjection, lipofection, and transfection employing polyamine transfection reagents. In one embodiment, the said vector is a recombinant baculovirus. In another embodiment, said recombinant baculovirus is transfected into a eukaryotic cell. In a preferred embodiment, said cell is an insect cell. In another embodiment, said insect cell is a Sf9 cell.

**[00125]** In another embodiment, said vector and/or host cell comprise nucleotides which encode an avian, pandemic and/or seasonal influenza virus HA protein selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15 and H16. In another embodiment, said vector and/or host cells comprise nucleotides which encode an NA protein which is selected from the group consisting of N1, N2, N3, N4, N5, N6, N7, N8 and N9. In another embodiment, said vector and/or host cell comprises influenza HA, M1 and/or NA. In another embodiment, said vector and/or host cell consists essentially of HA, M1 and/or NA. In a further embodiment, said vector and/or host cell consists of influenza protein comprising HA, M1 and NA. These vector and/or host cell contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, said nucleotides encode for an HA and/or the NA that exhibits hemagglutinin activity and/or neuraminidase activity, respectively, when expressed on the surface of VLPs.

[00126] This invention also provides for constructs and methods that will increase the efficiency of VLPs production. For example, removing cleavage sites from proteins in order to increase protein expression (see above). Other method comprises the addition of leader sequences to the HA, NA and/or M1 protein for more efficient transporting. For example, a heterologous signal sequence can be fused to the HA, NA and/or M1 influenza protein. In one embodiment, the signal sequence can be derived from the gene of an insect cell and fused to the influenza HA protein (for expression in insect cells). In another embodiment, the signal peptide is the chitinase signal sequence, which works efficiently in baculovirus expression systems. In other embodiment, interchanging leader sequences between influenza proteins can provide better protein transport. For example, it has been shown that H5 hemagglutinin is less efficient at being transported to the surface of particles. H9 hemagglutinins, however, targets the surface and is integrated into the surface more efficiently. Thus, in one embodiment, the H9 leader sequence is fused to the H5 protein.

[00127] Another method to increase efficiency of VLP production is to codon optimize the nucleotides that encode HA, NA and/or M1 proteins for a specific cell type. For example, codon optimizing nucleic acids for expression in Sf9 cell (see U.S. patent publication 2005/0118191, herein incorporated by reference in its entirety for all purposes). Examples of optimized codon sequences for Sf9 cells are disclosed below (e.g. SEQ ID 42, 44, 46, 48, 50, 52, and 54). In one embodiment, the nucleic acid sequence of codon optimized influenza protein is at least 85%, 90%, 95%, 96, 97, 98, or 99% to any one of SEQ ID Nos. 42, 44, 46, 48, 50, 52, and 54.

[00128] The invention also provides for methods of producing VLPs, said methods comprising expressing an avian, pandemic and/or seasonal influenza proteins under conditions that allow VLP formation. Depending on the expression system and host cell selected, the VLPs are produced by growing host cells transformed by an expression vector under conditions whereby the recombinant proteins are expressed and VLPs are formed. The selection of the appropriate growth conditions is within the skill or a person with skill of one of ordinary skill in the art.

[00129] Methods to grow cells engineered to produce VLPs of the invention include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. Cell culture means the growth and propagation of cells in a bioreactor (a fermentation chamber)

where cells propagate and express protein (*e.g.* recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions in a bioreactor. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, said bioreactor is a stainless steel chamber. In another embodiment, said bioreactor is a pre-sterilized plastic bag (*e.g.* Cellbag®, Wave Biotech, Bridgewater, NJ). In other embodiment, said pre-sterilized plastic bags are about 50 L to 1000 L bags.

**[00130]** The VLPs are then isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, *e.g.*, cesium chloride, sucrose and iodixanol, as well as standard purification techniques including, *e.g.*, ion exchange and gel filtration chromatography.

**[00131]** The following is an example of how VLPs of the invention can be made, isolated and purified. Usually VLPs are produced from recombinant cell lines engineered to create a VLP when said cells are grown in cell culture (see above). Production of VLPs may be accomplished by the scheme illustrated in Figure 26. A person of skill in the art would understand that there are additional methods that can be utilized to make and purify VLPs of the invention, thus the invention is not limited to the method described.

**[00132]** Production of VLPs of the invention can start by seeding Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125-ml flask to a 50 L Wave bag). The medium used to grow the cell is formulated for the appropriate cell line (preferably serum free media, *e.g.* insect medium ExCell-420, JRH). Next, said cells are infected with recombinant baculovirus at the most efficient multiplicity of infection (*e.g.* from about 1 to about 3 plaque forming units per cell). Once infection has occurred, the influenza HA, NA and M1 proteins are expressed from the virus genome, self assemble into VLPs and are secreted from the cells approximately 24 to 72 hours post infection. Usually, infection is most efficient when the cells are in mid-log phase of growth (4-8 $\times 10^6$ cells/ml) and are at least about 90% viable.

**[00133]** VLPs of the invention can be harvested approximately 48 to 96 hours post infection, when the levels of VLPs in the cell culture medium are near the maximum but before extensive cell lysis. The Sf9 cell density and viability at the time of harvest can be about $0.5\times 10^6$ cells/ml to about $1.5 \times 10^6$ cells/ml with at least 20% viability, as shown by dye exclusion assay. Next, the medium is removed and clarified. NaCl can be added to the medium to a concentration of about 0.4 to about 1.0 M, preferably to about 0.5 M, to avoid

VLP aggregation. The removal of cell and cellular debris from the cell culture medium containing VLPs of the invention can be accomplished by tangential flow filtration (TFF) with a single use, pre-sterilized hollow fiber 0.5 or 1.00 μm filter cartridge or a similar device.

[00134] Next, VLPs in the clarified culture medium can be concentrated by ultrafiltration using a disposable, pre-sterilized 500,000 molecular weight cut off hollow fiber cartridge. The concentrated VLPs can be diafiltrated against 10 volumes pH 7.0 to 8.0 phosphate-buffered saline (PBS) containing 0.5 M NaCl to remove residual medium components.

[00135] The concentrated, diafiltered VLPs can be furthered purified on a 20% to 60% discontinuous sucrose gradient in pH 7.2 PBS buffer with 0.5 M NaCl by centrifugation at 6,500 × g for 18 hours at about 4° C to about 10° C. Usually VLPs will form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that can be collected from the gradient and stored. This product can be diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. This product contains VLPs and may contain intact baculovirus particles.

[00136] Further purification of VLPs can be achieved by anion exchange chromatography, or 44% isopycnic sucrose cushion centrifugation. In anion exchange chromatography, the sample from the sucrose gradient (see above) is loaded into column containing a medium with an anion (e.g. Matrix Fractogel EMD TMAE) and eluded via a salt gradient (from about 0.2 M to about 1.0 M of NaCl) that can separate the VLP from other contaminates (e.g. baculovirus and DNA/RNA). In the sucrose cushion method, the sample comprising the VLPs is added to a 44% sucrose cushion and centrifuged for about 18 hours at 30,000 g. VLPs form a band at the top of 44% sucrose, while baculovirus precipitates at the bottom and other contaminating proteins stay in the 0% sucrose layer at the top. The VLP peak or band is collected.

[00137] The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or β-propyl lactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the VLPs in 0.2% of BPL for 3 hours at about 25 °C to about 27 °C. The baculovirus can also be inactivated by incubating the sample containing the VLPs at 0.05% BPL at 4 °C for 3 days, then at 37 °C for one hour.

[00138] After the inactivation/removal step, the product comprising VLPs can be run through another diafiltration step to remove any reagent from the inactivation step and/or any residual sucrose, and to place the VLPs into the desired buffer (*e.g.* PBS). The solution comprising VLPs can be sterilized by methods known in the art (*e.g.* sterile filtration) and stored in the refrigerator or freezer.

[00139] The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre-sterilized plastic bag (for example, the system sold by Wave Biotech, Bridgewater, NJ). A person with skill in the art will know what is most desirable for their purposes.

[00140] Expansion and production of baculovirus expression vectors and infection of cells with recombinant baculovirus to produce recombinant influenza VLPs can be accomplished in insect cells, for example Sf9 insect cells as previously described. In a preferred embodiment, the cells are SF9 infected with recombinant baculovirus engineered to produce influenza VLPs.

**Pharmaceutical or Vaccine Formulations and Administration**

[00141] The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition, and which may be administered without undue toxicity and a VLP of the invention. As used herein, the term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopia, European Pharmacopia or other generally recognized pharmacopia for use in vertebrates, and more particularly in humans. These compositions can be useful as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate.

[00142] Said pharmaceutical formulations of the invention comprise VLPs comprising an influenza M1, HA and/or NA protein and a pharmaceutically acceptable carrier or excipient. Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition).

The formulation should suit the mode of administration. In a preferred embodiment, the formulation is suitable for administration to humans, preferably is sterile, non-particulate and/or non-pyrogenic.

**[00143]** The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

**[00144]** The invention also provides for a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the vaccine formulations of the invention. In a preferred embodiment, the kit comprises two containers, one containing VLPs and the other containing an adjuvant. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

**[00145]** The invention also provides that the VLP formulation be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of composition. In one embodiment, the VLP composition is supplied as a liquid, in another embodiment, as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. Preferably, the VLP composition is supplied as a dry sterile lyophilized powder in a hermetically sealed container at a unit dosage of preferably, about 1 μg, about 5 μg, about 10 μg, about 20 μg, about 25 μg, about 30 μg, about 50 μg, about 100 μg, about 125 μg, about 150 μg, or about 200 μg. Alternatively, the unit dosage of the VLP composition is less than about 1 μg, (for example about 0.08 μg, about 0.04 μg ; about 0.2 μg, about 0.4 μg , about 0.8 μg , about 0.5 μg or less, about 0.25 μg or less, or about 0.1 μg or less), or more than about 125 μg, (for example about 150 μg or more, about 250 μg or more, or about 500 μg or more). These doses may be measured as total VLPs or as μg of HA. The VLP composition should be administered within about 12 hours, preferably within about 6 hours, within about 5 hours, within about 3 hours, or within about 1 hour after being reconstituted from the lyophylized powder.

**[00146]** In an alternative embodiment, a VLP composition is supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the VLP composition. Preferably, the liquid form of the VLP composition is supplied in a hermetically sealed container at least about 50 μg/ml, more preferably at least about 100 μg/ml, at least about 200 μg/ml, at least 500 μg /ml, or at least 1 mg/ml.

**[00147]** Generally, influenza VLPs of the invention are administered in an effective amount or quantity (as defined above) sufficient to stimulate an immune response against one or more strains of influenza virus. Preferably, administration of the VLP of the invention elicits substantial immunity against at least one influenza virus. Typically, the dose can be adjusted within this range based on, *e.g.*, age, physical condition, body weight, sex, diet, time of administration, and other clinical factors. The prophylactic vaccine formulation is systemically administered, *e.g.*, by subcutaneous or intramuscular injection using a needle and syringe, or a needle-less injection device. Alternatively, the vaccine formulation is administered intranasally, either by drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract. While any of the above routes of delivery results in an immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of the influenza virus.

**[00148]** Thus, the invention also comprises a method of formulating a vaccine or antigenic composition that induces substantial immunity to influenza virus infection or at least one symptom thereof to a subject, comprising adding to said formulation an effective dose of an influenza VLP.

**[00149]** While stimulation of substantial immunity with a single dose is preferred, additional dosages can be administered, by the same or different route, to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against influenza infection. Similarly, adults who are particularly susceptible to repeated or serious influenza infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

[00150] Thus, in one embodiment, a method to induce substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP comprises influenza HA, NA and M1 proteins. In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists essentially of influenza HA, NA and M1. Said VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists of influenza HA, NA and M1. In another embodiment, said influenza HA, NA and M1 is derived from seasonal influenza and/or avian influenza virus. In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In another embodiment, the method comprises inducing substantial immunity to influenza virus infection or at least one symptom thereof by administering said formulation in one dose. In another embodiment, the method comprises inducing substantial immunity to influenza virus infection or at least one symptom thereof by administering said formulation in multiple doses.

[00151] Methods of administering a composition comprising VLPs (vaccine and/or antigenic formulations) include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral or pulmonary routes or by suppositories). In a specific embodiment, compositions of the present invention are administered intramuscularly, intravenously, subcutaneously, transdermally or intradermally. The compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucous, colon, conjunctiva, nasopharynx,

oropharynx, vagina, urethra, urinary bladder and intestinal mucosa, etc.) and may be administered together with other biologically active agents. In some embodiments, intranasal or other mucosal routes of administration of a composition comprising VLPs of the invention may induce an antibody or other immune response that is substantially higher than other routes of administration. In another embodiment, intranasal or other mucosal routes of administration of a composition comprising VLPs of the invention may induce an antibody or other immune response that will induce cross protection against other strains of influenza viruses. Administration can be systemic or local.

**[00152]** In yet another embodiment, the vaccine and/or antigenic formulation is administered in such a manner as to target mucosal tissues in order to elicit an immune response at the site of immunization. For example, mucosal tissues such as gut associated lymphoid tissue (GALT) can be targeted for immunization by using oral administration of compositions which contain adjuvants with particular mucosal targeting properties. Additional mucosal tissues can also be targeted, such as nasopharyngeal lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

**[00153]** Vaccines and/or antigentic formulations of the invention may also be administered on a dosage schedule, for example, an initial administration of the vaccine composition with subsequent booster administrations. In particular embodiments, a second dose of the composition is administered anywhere from two weeks to one year, preferably from about 1, about 2, about 3, about 4, about 5 to about 6 months, after the initial administration. Additionally, a third dose may be administered after the second dose and from about three months to about two years, or even longer, preferably about 4, about 5, or about 6 months, or about 7 months to about one year after the initial administration. The third dose may be optionally administered when no or low levels of specific immunoglobulins are detected in the serum and/or urine or mucosal secretions of the subject after the second dose. In a preferred embodiment, a second dose is administered about one month after the first administration and a third dose is administered about six months after the first administration. In another embodiment, the second dose is administered about six months after the first administration.

**[00154]** In another embodiment, said VLP of the invention can be administered as part of a combination therapy. For example, VLPs of the invention can be formulated with other immunogenic compositions and/or antivirals (*e.g.* Amantadine, Rimantadine, Zanamivir and Osteltamivir).

[00155] The dosage of the pharmaceutical formulation can be determined readily by the skilled artisan, for example, by first identifying doses effective to elicit a prophylactic or therapeutic immune response, *e.g.*, by measuring the serum titer of virus specific immunoglobulins or by measuring the inhibitory ratio of antibodies in serum samples, or urine samples, or mucosal secretions. Said dosages can be determined from animal studies. A non-limiting list of animals used to study the influenza virus include the guinea pig, Syrian hamster, chinchilla, hedgehog, chicken, rat, mouse and ferret. Most animals are not natural hosts to influenza viruses but can still serve in studies of various aspects of the disease. For example, any of the above animals can be dosed with a vaccine candidate, *e.g.* VLPs of the invention, to partially characterize the immune response induced, and/or to determine if any neutralizing antibodies have been produced. For example, many studies have been conducted in the mouse model because mice are small size and their low cost allows researchers to conduct studies on a larger scale. Nevertheless, the mouse's small size also increases the difficulty of readily observing any clinical signs of the disease and the mouse is not a predictive model for disease in humans.

[00156] There has been extensive use of ferrets for studying various aspects of human influenza viral infection and its course of action. The development of many of the contemporary concepts of immunity to the influenza virus would have been impossible without the use of the ferret (Maher *et al.* 2004). Ferrets have proven to be a good model for studying influenza for several reasons: influenza infection in the ferret closely resembles that in humans with respect to clinical signs, pathogenesis, and immunity; types A and B of human influenza virus naturally infect the ferret, thus providing an opportunity to study a completely controlled population in which to observe the interplay of transmission of infection, illness, and sequence variation of amino acids in the glycoproteins of the influenza virus; and ferrets have other physical characteristics that make it an ideal model for deciphering the manifestations of the disease. For example, ferrets and humans show very similar clinical signs of influenza infection that seem to depend on the age of the host, the strain of the virus, environmental conditions, the degree of secondary bacterial infection, and many other variables. Thus, one skilled in the art can more easily correlate the efficacy of an influenza vaccine and dosage regiments from a ferret model to humans as compared to a mouse or any other model described above.

[00157] In addition, human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well

known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal test systems.

**[00158]** As also well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens (*e.g.*, U.S. Pat. No. 4,877,611). Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. The inclusion of any adjuvant described in Vogel *et al.*, "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes, is envisioned within the scope of this invention.

**[00159]** Exemplary, adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed Mycobacterium tuberculosis), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MF-59, Novasomes®, MHC antigens may also be used.

**[00160]** In one embodiment of the invention the adjuvant is a paucilamellar lipid vesicle having about two to ten bilayers arranged in the form of substantially spherical shells separated by aqueous layers surrounding a large amorphous central cavity free of lipid bilayers. Paucilamellar lipid vesicles may act to stimulate the immune response several ways, as non-specific stimulators, as carriers for the antigen, as carriers of additional adjuvants, and combinations thereof. Paucilamellar lipid vesicles act as non-specific immune stimulators when, for example, a vaccine is prepared by intermixing the antigen with the preformed vesicles such that the antigen remains extracellular to the vesicles. By encapsulating an antigen within the central cavity of the vesicle, the vesicle acts both as an immune stimulator and a carrier for the antigen. In another embodiment, the vesicles are primarily made of nonphospholipid vesicles. In other embodiment, the vesicles are

Novasomes. Novasomes® are paucilamellar nonphospholipid vesicles ranging from about 100 nm to about 500 nm. They comprise Brij 72, cholesterol, oleic acid and squalene. Novasomes have been shown to be an effective adjuvant for influenza antigens (*see*, U.S. Patents 5,629,021, 6,387,373, and 4,911,928, herein incorporated by reference in their entireties for all purposes).

**[00161]** In one aspect, an adjuvant effect is achieved by use of an agent, such as alum, used in about 0.05 to about 0.1% solution in phosphate buffered saline. Alternatively, the VLPs can be made as an admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution. Some adjuvants, for example, certain organic molecules obtained from bacteria; act on the host rather than on the antigen. An example is muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine [MDP]), a bacterial peptidoglycan. In other embodiments, hemocyanins and hemoerythrins may also be used with VLPs of the invention. The use of hemocyanin from keyhole limpet (KLH) is preferred in certain embodiments, although other molluscan and arthropod hemocyanins and hemoerythrins may be employed.

**[00162]** Various polysaccharide adjuvants may also be used. For example, the use of various pneumococcal polysaccharide adjuvants on the antibody responses of mice has been described (Yin *et al.*, 1989). The doses that produce optimal responses, or that otherwise do not produce suppression, should be employed as indicated (Yin *et al.*, 1989). Polyamine varieties of polysaccharides are particularly preferred, such as chitin and chitosan, including deacetylated chitin. In another embodiment, a lipophilic disaccharide-tripeptide derivative of muramyl dipeptide which is described for use in artificial liposomes formed from phosphatidyl choline and phosphatidyl glycerol.

**[00163]** Amphipathic and surface active agents, *e.g.*, saponin and derivatives such as QS21 (Cambridge Biotech), form yet another group of adjuvants for use with the VLPs of the invention. Nonionic block copolymer surfactants (Rabinovich *et al.*, 1994) may also be employed. Oligonucleotides are another useful group of adjuvants (Yamamoto *et al.*, 1988). Quil A and lentinen are other adjuvants that may be used in certain embodiments of the present invention.

**[00164]** Another group of adjuvants are the detoxified endotoxins, such as the refined detoxified endotoxin of U.S. Pat. No. 4,866,034. These refined detoxified endotoxins are effective in producing adjuvant responses in vertebrates. Of course, the detoxified endotoxins may be combined with other adjuvants to prepare multi-adjuvant formulation. For example, combination of detoxified endotoxins with trehalose dimycolate is particularly contemplated, as described in U.S. Pat. No. 4,435,386. Combinations of detoxified endotoxins with trehalose dimycolate and endotoxic glycolipids is also contemplated (U.S. Pat. No. 4,505,899), as is combination of detoxified endotoxins with cell wall skeleton (CWS) or CWS and trehalose dimycolate, as described in U.S. Pat. Nos. 4,436,727, 4,436,728 and 4,505,900. Combinations of just CWS and trehalose dimycolate, without detoxified endotoxins, is also envisioned to be useful, as described in U.S. Pat. No. 4,520,019.

**[00165]** Those of skill in the art will know the different kinds of adjuvants that can be conjugated to vaccines in accordance with this invention and these include alkyl lysophosphilipids (ALP); BCG; and biotin (including biotinylated derivatives) among others. Certain adjuvants particularly contemplated for use are the teichoic acids from Gram-cells. These include the lipoteichoic acids (LTA), ribitol teichoic acids (RTA) and glycerol teichoic acid (GTA). Active forms of their synthetic counterparts may also be employed in connection with the invention (Takada *et al.*, 1995).

**[00166]** Various adjuvants, even those that are not commonly used in humans, may still be employed in other vertebrates, where, for example, one desires to raise antibodies or to subsequently obtain activated T cells. The toxicity or other adverse effects that may result from either the adjuvant or the cells, *e.g.*, as may occur using non-irradiated tumor cells, is irrelevant in such circumstances.

**[00167]** Another method of inducing an immune response can be accomplished by formulating the VLPs of the invention with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules can be administered in the same formulation as the influenza VLPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

## Method of Stimulating an Anti-Influenza Immune Response

[00168] The VLPs of the invention are useful for preparing compositions that stimulate an immune response that confers immunity or substantial immunity to influenza viruses. Both mucosal and cellular immunity may contribute to immunity to influenza infection and disease. Antibodies secreted locally in the upper respiratory tract are a major factor in resistance to natural infection. Secretory immunoglobulin A (sIgA) is involved in protection of the upper respiratory tract and serum IgG in protection of the lower respiratory tract. The immune response induced by an infection protects against reinfection with the same virus or an antigenically similar viral strain. Influenza virus undergoes frequent and unpredictable changes; therefore, after natural infection, the effective period of protection provided by the host's immunity may only be a few years against the new strains of virus circulating in the community.

[00169] VLPs of the invention can induce substantial immunity in a vertebrate (*e.g.* a human) when administered to said vertebrate. The substantial immunity results from an immune response against the influenza VLP of the invention that protects or ameleorates influenza infection or at least reduces a symptom of influenza virus infection in said vertebrate. In some instances, if the said vertebrate is infected, said infection will be asymptomatic. The response may be not a fully protective response. In this case, if said vertebrate is infected with an influenza virus, the vertebrate will experience reduced symptoms or a shorter duration of symptoms compared to a non-immunized vertebrate.

[00170] In one embodiment, the invention comprises a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an influenza VLP. In another embodiment, said induction of substantial immunity reduces duration of influenza symptoms. In another embodiment, a method to induce substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP comprises influenza HA, NA and M1 proteins. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP

consists essentially of influenza HA, NA and M1. Said VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists of influenza HA, NA and M1. In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In a further embodiment, said VLP is formulated with an adjuvant or immune stimulator.

**[00171]** Recently there has been a concerted effort to create a vaccine against avian influenza virus that has the potential to create a pandemic. That is because a number of avian influenza viruses have crossed the species barrier and directly infected humans resulting in illness and, in some cases, death. These viruses were H5N1, H9N2 and H7N7 (Cox *et al.*, 2004). A recent study examined the potential of using inactivated H5N1 influenza virus as a vaccine. The formulation of the vaccine was similar to the licensed inactivated vaccines currently licensed for marketing. The study concluded that using inactivated H5N1 virus did induce an immune response in humans, however the dose given was very high (90 μg of avian influenza compared to 15 μg of the licensed vaccine) (Treanor *et al.*, 2006). This high amount of avian influenza antigen is impractical for a worldwide vaccination campaign. As illustrated below, the VLPs of the invention induces an immune response in a vertebrate when administered to said vertebrate.

**[00172]** Thus, the invention encompasses a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an avian influenza VLP. In another embodiment, said induction of substantial immunity reduces duration of influenza symptoms. In another embodiment, said induction of immunity is from administering at least 0.2 μg of avian HA in VLPs of the invention. In another embodiment, said induction of immunity is from administering about 0.2 μg of avian HA to about 15 μg of avian HA in VLPs of the invention. Administration may be in one or more doses, but may be advantageously in a single dose. In another embodiment, said VLP avian HA is derived from avian influenza H5N1.

**[00173]** In another embodiment, the invention comprises a method of inducing substantial immunity to avian influenza virus infection or at least one symptom thereof in a subject comprising administering at least one effective dose of an avian influenza VLP, wherein said VLP comprises an avian influenza HA, NA and M1. In another embodiment, said avian influenza VLP comprises avian influenza proteins, wherein said avian influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc. but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, said method of inducing substantial immunity to avian influenza virus infection or at least one symptom thereof in a subject comprises administering at least one effective dose of an avian influenza VLP, wherein said VLP consists essentially of avian influenza HA, NA and M1. Said VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, a method to induce substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists of avian influenza HA, NA and M1. In another embodiment, said avian influenza HA and NA are H5N1, respectively. In another embodiment, said avian influenza HA and NA are H9N2, respectively. In another embodiment, said avian influenza HA and NA are H7N7, respectively. In another embodiment, said avian influenza HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In a further embodiment, said VLP is formulated with an adjuvant or immune stimulator.

**[00174]** In another embodiment, said avian influenza VLPs will induce an immune response in a vertebrate that is about 2 fold, about 4 fold, about 8 fold, about 16 fold, about 32 fold about 64 fold, about 128 fold increase (or higher) more potent than a similar avian influenza antigens formulated similarly to the licensed inactivated vaccines currently licensed for marketing. Current formulations comprise whole inactivated virus (*e.g.* formaldehyde treated), split virus (chemically disrupted), and subunit (purified glycoprotein) vaccines. Methods for determining potency for a vaccine are known and routine in the art. For example, microneutralization assays and hemagglutination inhibition assays can be performed to determine potency of an avian VLP vaccine compared to avian influenza antigens formulated similar to the licensed inactivated vaccines currently licensed for marketing. In one embodiment, said increase in potency is realized when about 0.2 μg , about 0.4 μg , about 0.6 μg about 0.8 μg, about 1 μg, about 2 μg, about 3 μg, about 4 μg, about 5 μg, about 6 μg, about 7 μg, about 9 μg, about 10 μg, about 15 μg, about 20 μg, about 25 μg, about 30 μg, about 35 μg, 40 μg, about 45 μg, about 50 μg, or higher of VLPs and the antigen formulated similarly to the inactivated vaccines currently licensed for marketing is administered to a vertebrate (*i.e.* equivalent amounts of HA and/or NA in a VLP with equivalent amounts of HA and/or NA formulated in similarly to the licensed inactivated vaccines and/or any other antigen) Amounts can be measured according to HA content. For example, 1 μg of a VLP of the invention is about 1 μg of HA in a solution of VLPs comprising HA or may be measured by weight of VLPs.

**[00175]** Seasonal influenza vaccines are administered to humans every year to reduce the incidence of influenza cases every year. At present, there are two subtypes of influenza A and influenza B circulating in the United States. Current vaccines are, therefore, trivalent to provide protection against the strains currently circulating. Each year a different stain or variation of an influenza viral changes. Thus, for most years a new vaccine composition is manufactured and administered. Inactivated vaccines are produced by propagation of the virus in embryonated hens' eggs. The allantoic fluid is harvested, and the virus is concentrated and purified, then inactivated. Thus, the current licensed influenza virus vaccines may contain trace amounts of residual egg proteins and, therefore, should not be administered to persons who have anaphylactic hypersesitiviety to eggs. In addition, supplies of eggs must be organized and strains for vaccine production must be selected months in advance of the next influenza season, thus limiting the flexibility of this approach and often resulting in delays and shortages in production and distribution. In addition, some influenza strains do not replicate well in embryonated chicken eggs which may limit the influenza strains which can be grown and formulated into vaccines.

**[00176]** As mentioned above, VLP of the invention do not require eggs for production. These VLPs are made *via* a cell culture system. Thus, the invention encompasses a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a seasonal influenza VLP. A discussed above, seasonal influenza virus refers to the influenza viral strains that has been determined to be passing within the human population for a given influenza season based on the epidemiological surveys by National Influenza Centers worldwide. Said studies and some isolated influenza viruses are sent to one of four World Health Organization (WHO) reference laboratories, one of which is located at the Centers for Disease Control and Prevention (CDC) in Atlanta, for detailed testing. These laboratories test how well antibodies made to the current vaccine react to the circulating virus and new flu viruses. This information, along with information about flu activity, is summarized and presented to an advisory committee of the U.S. Food and Drug Administration (FDA) and at a WHO meeting. These meetings result in the selection of three viruses (two subtypes of influenza A viruses and one influenza B virus) to go into flu vaccines for the following fall and winter. The selection occurs in February for the northern hemisphere and in September for the southern hemisphere. Usually, one or two of the three virus strains in the vaccine changes each year. In another embodiment, said induction of substantial immunity reduces duration of influenza symptoms.

[00177] In another embodiment, the invention comprises a method of inducing substantial immunity to a seasonal influenza virus infection or at least one symptom thereof in a subject comprising administering at least one effective dose of a seasonal influenza VLP, wherein said VLP comprises a seasonal influenza HA, NA and M1. In another embodiment, said seasonal influenza VLP comprises seasonal influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc. but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, said method of inducing substantial immunity to seasonal influenza virus infection or at least one symptom thereof in a subject comprises administering at least one effective dose of a seasonal influenza VLP, wherein said VLP consists essentially of seasonal influenza HA, NA and M1. Said VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, a method to induce substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists of seasonal influenza HA, NA and M1. In another embodiment, said avian influenza HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In a further embodiment, said VLP is formulated with an adjuvant or immune stimulator.

[00178] Generally, seasonal influenza VLPs of the invention are administered in a quantity sufficient to stimulate substantial immunity for one or more strains of seasonal influenza virus. In one embodiment, the VLPs are blended together with other VLPs comprising different influenza subtypes proteins (as listed above). In another embodiment, the

formulation is a trivalent formulation which comprises a mixture of VLPs with seasonal influenza HA and/or NA proteins from at least two influenza A and/or one at least one B subtype. In another embodiment, said B subtype is produced by the same method as described above. In another embodiment, a multivalent formulation comprises one or more of the VLP of the invention as described above.

**[00179]** In another embodiment, VLPs of the invention (avian or seasonal VLPs) may elicit an immune response that will provide protection against more than one strain of influenza virus. This cross-protection of a vertebrate with an influenza VLP constructed from a particular strain, of a particular subgroup, may induce cross-protection against influenza virus of different strains and/or subgroups. The examples below show that VLPs of the invention are capable of inducing cross reactivity with different strains and/or subgroups.

**[00180]** The humoral immune system produces antibodies against different influenza antigens, of which the HA-specific antibody is the most important for neutralization of the virus and thus prevention of illness. The NA-specific antibodies are less effective in preventing infection, but they lessen the release of virus from infected cells. The mucosal tissues are the main portal entry of many pathogens, including influenza, and the mucosal immune system provides the first line of defense against infection apart from innate immunity. SIgA and, to some extent, IgM are the major neutralizing antibodies directed against mucosal pathogens preventing pathogen entry and can function intracellularly to inhibit replication of virus. Nasal secretions contain neutralizing antibodies particularly to influenza HA and NA, which are primarily of the IgA isotype and are produced locally. During primary infection, all three major Ig classes (IgG, IgA and IgM) specific to HA can be detected by enzyme-linked immunosorbent assay in nasal washings, although IgA and IgM are more frequently detected than IgG. Both IgA and, to some extent, IgM are actively secreted locally, whereas IgG is derived as a serum secretion. In subjects who have a local IgA response, a serum IgA response also is observed. The local IgA response stimulated by natural infection lasts for at least 3–5 months, and influenza-specific, IgA-committed memory cells can be detected locally. IgA also is the predominant Ig isotype in local secretions after secondary infection, and an IgA response is detected in the serum upon subsequent infection. The presence of locally produced neutralizing antibodies induced by live virus vaccine correlates with resistance to infection and illness after challenge with wild-type virus.

[00181] Resistance to influenza infection or illness is correlated with the level of local and/or serum antibody to HA and NA. Serum anti-HA antibodies are the most commonly measured correlate of protection against influenza (Cox *et al.*, 1999). A protective serum antibody (haemagglutination inhibition (HI) titer $\geq$ 40) response can be detected in approximately 80% of subjects after natural influenza infection. B cells producing all three major Ig classes are present in the peripheral blood in normal subjects (Cox *et al.*, 1994) and individuals undergoing influenza infection. In humans, serum antibodies play a role in both resistance to and recovery from influenza infection. The level of serum antibody to HA and NA in humans can be correlated with resistance to illness following experimental infection and natural infection. During primary infection, the three major Ig classes can be detected within 10–14 days. IgA and IgM levels peak after 2 weeks and then begin to decline, whereas the level of IgG peaks at 4–6 weeks. Whereas IgG and IgM are dominant in the primary response, IgG and IgA predominate in the secondary immune response.

[00182] Thus, the invention encompasses a method of inducing a substantially protective antibody response to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an influenza VLP. In another embodiment, said induction of substantially protective antibody response reduces duration of influenza symptoms. In another embodiment, a method to induce substantially protective antibody response to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP comprises influenza HA, NA and M1 proteins.

[00183] In another embodiment, the invention comprises a method of inducing substantially protective antibody response to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists essentially of influenza HA, NA and M1. Said VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP

consists of influenza HA, NA and M1. In another embodiment, wherein said influenza HA, NA and M1 is derived from seasonal influenza and/or avian influenza. In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In a further embodiment, said VLP is formulated with an adjuvant or immune stimulator.

**[00184]** As used herein, an "antibody" is a protein comprising one or more polypeptides substantially or partially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. A typical immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. Antibodies exist as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases.

**[00185]** Cell-mediated immunity also plays a role in recovery from influenza infection and may prevent influenza-associated complications. Influenza-specific cellular lymphocytes have been detected in the blood and the lower respiratory tract secretions of infected subjects. Cytolysis of influenza-infected cells is mediated by CTLs in concert with influenza-specific antibodies and complement. The primary cytotoxic response is detectable in blood after 6–14 days and disappears by day 21 in infected or vaccinated individuals (Ennis *et al.,* 1981). Influenza-specific CTLs exhibit cross-reactive specificities in *in vitro* cultures; thus, they lyse cells infected with the same type of influenza but not with other types (*e.g.* influenza A but not influenza B virus). CTLs that recognize the internal nonglycosylated proteins, M, NP and PB2 have been isolated (Fleischer *et al.*, 1985). The CTL response is cross-reactive between influenza A strains (Gerhard *et al.*, 2001) and is important in minimizing viral spread in combination with antibody (Nguyen *et al.*, 2001).

**[00186]** Cell-mediated immunity also plays a role in recovery from influenza infection and may prevent influenza-associated complications. Influenza-specific cellular lymphocytes have been detected in the blood and the lower respiratory tract secretions of infected subjects. Cytolysis of influenza-infected cells is mediated by CTLs in concert with influenza-specific antibodies and complement. The primary cytotoxic response is detectable in blood after 6–14 days and disappears by day 21 in infected or vaccinated individuals (Ennis *et al.*, 1981). Influenza-specific CTLs exhibit cross-reactive specificities in *in vitro* cultures; thus, they lyse cells infected with the same type of influenza but not with other types (*e.g.* influenza A but not influenza B virus). CTLs that recognize the internal nonglycosylated proteins, M, NP and PB2 have been isolated(Fleischer *et al.*, (1985). The CTL response is cross-reactive between influenza A strains (Gerhard *et al.*, 2001) and is important in minimizing viral spread in combination with antibody (Nguyen *et al.*, 2001).

**[00187]** Thus, the invention encompasses a method of inducing a substantially protective cellular immune response to influenza virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of an influenza VLP. In another embodiment, a method of inducing substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of an influenza VLP, wherein said VLP consists of influenza HA, NA and M1. In another embodiment, said influenza VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc. but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment wherein said influenza HA, NA and M1 is derived from seasonal influenza and/or avian influenza virus. In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In a further embodiment, said VLP is formulated with an adjuvant or immune stimulator.

**[00188]** As mentioned above, the VLPs of the invention (*e.g.* avian and/or seasonal influenza VLPs) prevent or reduce at least one symptom of influenza infection in a subject. Symptoms of influenza are well known in the art. They include fever, myalgia, headache, severe malaise, nonproductive cough, sore throat, weight loss and rhinitis. Thus, the method of the invention comprises the prevention or reduction of at least one symptom associated with influenza viral infection. A reduction in a symptom may be determined subjectively or objectively, *e.g.*, self assessment by a subject, by a clinician's assessment or by conducting an appropriate assay or measurement (*e.g.* body temperature), including, *e.g.*, a quality of life assessment, a slowed progression of an influenza infection or additional symptoms, a reduced severity of a influenza symptoms or a suitable assays (*e.g.* antibody titer and/or T-cell activation assay). The objective assessment comprises both animal and human assessments.

**[00189]** The principal strategy advocated by the Advisory Committee on Immunization Practices (ACIP) for control of influenza has been the vaccination of persons at risk for serious complications from influenza, in particular, people ≥65 years old. Yearly influenza epidemics, however, continue unabated and are responsible for significant health and financial burden to our society (Glaser *et al.*, 1996). In the last 20 years (1976–1999), a significant increase has occurred in influenza-associated all cause excess deaths. From 1990 to 1999, the annual number of influenza-associated all cause deaths exceeded 50,000 (Thompson *et al.*, 2003). Despite the increase in vaccine coverage of people ≥ 65 years to 65% during the last decade, a corresponding reduction in influenza-associated all cause excess deaths has not been observed.

**[00190]** Thus, another strategy for the prevention and control of influenza is universal vaccination of healthy children and individuals. Children have high rates of infection, medically attended illness and hospitalization from influenza (Neuzil *et al.,* 2000). Children play an important role in the transmission of influenza within schools, families and communities. Vaccination with current influenza vaccines of approximately 80% of schoolchildren in a community has decreased respiratory illnesses in adults and excess deaths in the elderly (Reichert *et al.*, 2001). This concept is known as community immunity or "herd immunity" and is thought to play an important part of protecting the community against disease. Because vaccinated people have antibodies that neutralize influenza virus, they are much less likely to transmit influenza virus to other people. Thus, even people who have not been vaccinated (and those whose vaccinations have become weakened or whose vaccines are not fully effective) often can be shielded by the herd immunity because vaccinated people around them are not getting sick. Herd immunity is more effective as the percentage of people vaccinated increases. It is thought that approximately 95% of the people in the community must be protected by a vaccine to achieve herd immunity. People who are not immunized increase the chance that they and others will get the disease.

**[00191]** Thus, the invention encompasses a method of inducing a substantially protective immunity to influenza virus infection to a population or a community in order to reduce the incidence of influenza virus infections among immunocompromised individuals or non-vaccinated individual buy administering VLPs of the invention to a population in a community. In one embodiment, most school-aged children are immunized against influenza virus by administering the VLPs of the invention. In another embodiment, most healthy individuals in a community to are immunized against influenza virus by administering the VLPs of the invention. In another embodiment VLPs of the invention are part of a "dynamic vaccination" strategy. Dynamic vaccination is the steady production of a low-efficacy vaccine that is related to an emerging pandemic strain, but due to an antigentic drift may not provide complete protection in a mammal (see Germann *et al.*, 2006). Because of the uncertainty about the future identity of a pandemic strain, it is almost impossible to stockpile a well matched pandemic strain. However, vaccination with a poorly matched but potentially efficacious vaccine may slow the spread of the pandemic virus and/or reduce the severity of symptoms of a pandemic strain of influenza virus.

**[00192]** The invention also encompasses a vaccine comprising an influenza VLP, wherein said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof when administered to a subject. In another embodiment, said induction of substantial immunity reduces duration of influenza symptoms. In another embodiment, a said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises a VLP which comprises influenza HA, NA and M1 proteins. In another embodiment, a said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises a VLP which consists essentially of influenza HA, NA and M1 proteins. Said VLPs may comprise additional influenza proteins and/or protein contaminates in negligible concentrations. In another embodiment, a said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises a VLP which consists of influenza HA, NA and M1 proteins. In another embodiment, a said vaccine induces substantial immunity to influenza virus infection or at least one symptom thereof in a subject, comprises a VLP comprises influenza proteins, wherein said influenza proteins consist of HA, NA and M1 proteins. These VLPs contain HA, NA and M1 and may contain additional cellular constituents such as cellular proteins, baculovirus proteins, lipids, carbohydrates etc., but do not contain additional influenza proteins (other than fragments of M1, HA and/or NA). In another embodiment, said influenza HA, NA and M1 proteins are derived from an avian and/or seasonal influenza virus. In another embodiment, said HA and/or NA exhibits hemagglutinin activity and/or neuraminidase activity, respectfully. In another embodiment, said subject is a mammal. In another embodiment, said mammal is a human. In a further embodiment, said VLP is formulated with an adjuvant or immune stimulator. In another embodiment, where said vaccine is administered to a mammal. In a further embodiment, said mammal is a human.

[00193] This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures and the Sequence Listing, are incorporated herein by reference.

## EXAMPLES

### Example 1

Materials and Methods

[00194] Avian influenza A/Hong Kong/1073/99 (H9N2) virus HA, NA, and M1 genes were expressed in Spodoptera frugiperda cells (Sf-9S cell line; ATCC PTA-4047) using the baculovirus bacmid expression system. The HA, NA, and M1 genes were synthesized by the reverse transcription and polymerase chain reaction (PCR) using RNA isolated from avian influenza A/Hong Kong/1073/99 (H9N2) virus (FIGS. 1, 2, and 3). For reverse transcription and PCR, oligonucleotide primers specific for avian influenza A/Hong Kong/1073/99 (H9N2) virus HA, NA, and M1 genes were used (Table 1). The cDNA copies of these genes were cloned initially into the bacterial subcloning vector, pCR2.1TOPO. From the resulting three pCR2.1TOPO-based plasmids, the HA, NA, and M1 genes were inserted downstream of the AcMNPV polyhedrin promoters in the baculovirus transfer vector, pFastBac1 (InVitrogen), resulting in three pFastBac1-based plasmids: pHA, pNA, and pM1 expressing these influenza virus genes, respectively. Then, a single pFastBac1-based plasmid pHAM was constructed encoding both the HA and M1 genes, each downstream from a separate polyhedrin promoter (FIG. 4). The nucleotide sequence of the NA gene with the adjacent 5'- and 3'-regions within the pNA plasmid was determined (SEQ ID NO:1) (FIG. 1). At the same time, the nucleotide sequences of the HA and M1 genes with the adjacent regions were also determined using the pHAM plasmid (SEQ ID NOS:2 and 3) (FIGS. 2 and 3).

[00195] Finally, a restriction DNA fragment from the pHAM plasmid that encoded both the HA and M1 expression cassettes was cloned into the pNA plasmid. This resulted in the

plasmid pNAHAM encoding avian influenza A/Hong Kong/1073/99 (H9N2) virus HA, NA, and M1 genes (FIG. 4).

**[00196]** Plasmid pNAHAM was used to construct a recombinant baculovirus containing influenza virus NA, HA, and M1 genes integrated into the genome, each downstream from a separate baculovirus polyhedrin promoter. Infection of permissive Sf-9S insect cells with the resulting recombinant baculovirus resulted in co-expression of these three influenza genes in each Sf-9S cell infected with such recombinant baculovirus.

**[00197]** The expression products in infected Sf-9S cells were characterized at 72 hr postinfection (p.i. by SDS-PAGE analysis, Coomassie blue protein staining, and Western immunoblot analysis using HA- and M1-specific antibodies (FIG. 5). Western immunoblot analysis was carried out using rabbit antibody raised against influenza virus type A/Hong Kong/1073/99 (H9N2) (CDC, Atlanta, Ga., USA), or mouse monoclonal antibody to influenza M1 protein (Serotec, UK). The HA, NA, and M1 proteins of the expected molecular weights (64 kd, 60 kd, and 31 kd, respectively) were detected by Western immunoblot analysis. Compared to the amount of HA protein detected in this assay, the NA protein showed lower reactivity with rabbit serum to influenza A/Hong Kong/1073/99 (H9N2) virus. Explanations for the amount of detectable NA protein included lower expression levels of the NA protein from Sf-9S cells infected with recombinant baculovirus as compared to the HA protein, lower reactivity of the NA with this serum under denaturing conditions in the Western immunoblot assay (due to the elimination of important NA epitopes during gel electrophoresis of membrane binding), lower NA-antibody avidity as compared to HA-antibody, or a lower abundance of NA-antibodies in the serum.

**[00198]** The culture medium from the Sf-9S cells infected with recombinant baculovirus expressing A/Hong Kong/1073/99 (H9N2) HA, NA, and M1 proteins was also probed for influenza proteins. The clarified culture supernatants were subjected to ultracentrifugation at 27,000 rpm in order to concentrate high-molecular protein complexes of influenza virus, such as subviral particles, VLP, complexes of VLP, and possibly, other self-assembled particulates comprised of influenza HA, NA, and M1 proteins. Pelleted protein products were resuspended in phosphate-buffered saline (PBS, pH 7.2) and further purified by ultracentrifugation on discontinuous 20-60% sucrose step gradients. Fractions from the sucrose gradients were collected and analyzed by SDS-PAGE analysis, Western immunoblot analysis, and electron microscopy.

[00199] Influenza HA and M1 proteins of the expected molecular weights were detected in multiple sucrose density gradient fractions by Coomassie blue staining and Western immunoblot analysis (FIG. 6, Table 1). This suggested that influenza viral proteins from infected Sf-9S cells are aggregated in complexes of high-molecular weight, such as capsomers, subviral particles, VLP, and/or VLP complexes. The NA proteins, although inconsistently detected by Coomassie blue staining and Western immunoblot analysis, which was likely due to the inability of the rabbit anti-influenza serum to recognize denatured NA protein in the Western immunoblot assay, were consistently detected in neuraminidase enzyme activity assay (FIG. 10).

TABLE 1

| Fraction#* | Titer |
|---|---|
| 1 | <1:5001 |
| 3 | <1:500 |
| 5 | 1:500 |
| 7 | 1:1000 |
| 9 | 1:2000 |
| 11 | 1:2000 |
| 12 | 1:4000 |
| 14 | 1:500 |
| 16 | <1:500 |
| PBS** | <1:500 |
| A/Shangdong/9/93 | <1:1000 |

*Fraction from 20-60% sucrose gradient
**Negative Control
***Positive Control

| Virus | Strain | Gene | RT-PCR Primer | | SEQ ID NO |
|---|---|---|---|---|---|
| Type A | (H3N2) Sydney/5/97 | Hemagglutinin (HA) | Forward | 5'-A GGATCC**ATG** AAGACTATCATTGCTTTGAG-3' | 13 |
| | | | Reverse | 5'-A GGTACC TCAAATGCAAATGTTGCACCTAATG-3' | 14 |
| | | Neuraminidase (NA) | Forward | 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGAAG GAGATAGAACC **ATG** AATCCAAATCAAAAGATAATAAC-3' | 15 |
| | | | Reverse | 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTATAT AGGCATGAGATTGATGTCCGC-3' | 16 |
| | | Matrix (M1) | Forward | 5'-AAA GAATTC **ATG** AGTCTTCTAACCGAGGTCGAAACGTA-3' | 17 |
| | | | Reverse | 5'-AAA TTCGAA TTACTCCAGCTCTATGCTGACAAAATGAC-3' | 18 |
| | | M2 | Forward | 5'-A GAATC **ATG** AGTCTTCTAACCGAGGTCGAAACGCCT ATCAGAAACGAATGGGGGTGC-3' | 19 |
| | | | Reverse | 5'-AAA TTCGAA TTACTCCAGCTCTATGCTGACAAAATGAC-3' | 20 |
| | | Nucleoprotein (NP) | Forward | 5'-A GAATTC **ATG** GCGTCCCAAGGCACCAAACG-3' | 21 |
| | | | Reverse | 5'-A GCGGCCGCTTAATTGTCGTACTCCTCTGCATTGTCTCCGAA GAAATAAG-3' | 22 |
| Type B | Harbin | Hemagglutinin (HA) | Forward | 5'-A GAATTC **ATG** AAGGCAATAATTGTACTACTCATGG-3' | 23 |
| | | | Reverse | 5'-A GCGGCCGCTTATAGACAGATGGAGCAAGAAACATTGTC TCTGGAGA-3' | 24 |
| | | Neuraminidase (NA) | Forward | 5'-A GAATT C**ATG** CTACCTTCAACTATACAAACG-3' | 25 |
| | | | Reverse | 5'-A GCGGCCGCTTACAGAGCCATATCAACACCTGTGACAGTG-3' | 26 |

[00200] The presence of high-molecular VLPs was confirmed by gel filtration chromatography. An aliquot from sucrose density gradient fractions containing influenza viral proteins was loaded onto a Sepharose CL-4B column for fractionation based on mass. The column was calibrated with dextran blue 2000, dextran yellow, and vitamin B12 (Amersham Pharmacia) with apparent molecular weights of 2,000,000; 20,000; and 1,357 daltons, respectively, and the void volume of the column was determined. As expected, high-molecular influenza viral proteins migrated in the void volume of the column, which was characteristic of macromolecular proteins, such as virus particles. Fractions were analyzed by Western immunoblot analysis to detect influenza and baculovirus proteins. For example, M1 proteins were detected in the void volume fractions, which also contained baculovirus proteins (FIG. 7).

[00201] The morphology of influenza VLPs and proteins in sucrose gradient fractions was elucidated by electron microscopy. For negative-staining electron microscopy, influenza proteins from two sucrose density gradient fractions were fixed with 2% glutaraldehyde in PBS, pH 7.2. Electron microscopic examination of negatively-stained samples revealed the presence of macromolecular protein complexes or VLPs in both fractions. These VLPs displayed different sizes including diameters of approximately 60 and 80 nm and morphologies (spheres). Larger complexes of both types of particles were also detected, as

well as rod-shaped particles (FIG. 8). All observed macromolecular structures had spikes (peplomers) on their surfaces, which is characteristic of influenza viruses. Since the size and appearance of 80 nm particles was similar to particles of wild type influenza virus, these structures likely represented VLPs, which have distinct similarities to wild type influenza virions, including similar particle geometry, architecture, triangulation number, symmetry, and other characteristics. The smaller particles of approximately 60 nm may represent subviral particles that differ from VLPs both morphologically and structurally. Similar phenomenon of recombinant macromolecular proteins of different sizes and morphologies was also reported for other viruses. For example, recombinant core antigen (HBcAg) of hepatitis B virus forms particles of different sizes, which have different architecture and triangulation number T=4 and T=3, respectively (Crowther *et al.*, 1994).

**[00202]** To characterize the functional properties of the purified influenza A/Hong Kong/1073/99 (H9N2) VLPs, samples were tested in a hemagglutination assay (FIG. 9) and a neuraminidase enzyme assay (FIG. 10). For the hemagglutination assay, 2-fold dilutions of purified influenza VLPs were mixed with 0.6% guinea pig red blood cells and incubated at 4° C. for 1 hr or 16 hr. The extent of hemagglutination was inspected visually and the highest dilution of recombinant influenza proteins capable of agglutinating red blood cells was determined and recorded (FIG. 9). Again, many fractions from the sucrose density gradient exhibited hemagglutination activity, suggesting that multiple macromolecular and monomeric forms of influenza proteins were present. The highest titer detected was 1:4000. In a control experiment, wild-type influenza A/Shangdong virus demonstrated a titer of 1:2000. The hemagglutination assay revealed that the recombinant VLPs consisting of influenza A/Hong Kong/1073/99 (H9N2) virus HA, NA, and M1 proteins were functionally active. This suggested that the assembly, conformation, and folding of the HA subunit proteins within the VLPs were similar or identical to that of the wild type influenza virus.

**[00203]** Additionally, a neuraminidase enzyme assay was performed on samples of purified H9N2 VLPs. The amount of neuraminidase activity in sucrose density gradient fractions was determined using fetuin as a substrate. In the neuraminidase assay, the neuraminidase cleaved sialic acid from substrate molecules to release sialic acid for measurement. Arsenite reagent was added to stop enzyme activity. The amount of sialic acid liberated was determined chemically with thiobarbituric acid that produces a pink color that was proportional to the amount of free sialic acid. The amount of color (chromophor) was measured spectrophotometrically at wavelength 549 nm. Using this method, neuraminidase activity was demonstrated in sucrose gradient fractions containing influenza VLPs (FIG. 10). As expected, the activity was observed in several fractions, with two peak fractions. As a positive control, wild type influenza virus was used. The wild type influenza virus exhibited neuraminidase enzyme activity comparable to that of purified influenza VLPs. These findings corroborated the HA results with regard to protein conformation and suggested that purified VLPs of influenza A/Hong Kong/1073/99 (H9N2) virus were functionally similar to wild type influenza virus.

**[00204]** The results from the above analyses and assays indicated that expression of influenza A/Hong Kong/1073/99 (H9N2) HA, NA, and M1 proteins was sufficient for the self-assembly and transport of functional VLPs from baculovirus-infected insect cells. Since these influenza VLPs represented self-assembled influenza structural proteins and demonstrated functional and biochemical properties similar to those of wild type influenza virus, these influenza VLPs conserved important structural conformations including surface epitopes necessary for effective influenza vaccines.

**Example 2**

**RT-PCR Cloning of Avian Influenza A/Hong Kong/1073/99 Viral Genes**

**[00205]** It is an object of the present invention to provide synthetic nucleic acid sequences capable of directing production of recombinant influenza virus proteins. Such synthetic nucleic acid sequences were obtained by reverse transcription and polymerase chain reaction (PCR) methods using influenza virus natural genomic RNA isolated from the virus. For the purpose of this application, nucleic acid sequence refers to RNA, DNA, cDNA or any synthetic variant thereof which encodes the protein.

**[00206]** Avian influenza A/Hong Kong/1073/99 (H9N2) virus was provided by Dr. K. Subbarao (Centers for Disease Control, Atlanta, Ga., USA). Viral genomic RNA was isolated by the acid phenol RNA extraction method under Biosafety Level 3 (BSL3) containment conditions at CDC using Trizol LS reagent (Invitrogen, Carlsbad, Calif. USA). cDNA molecules of the viral RNAs were obtained by reverse transcription using MuLV reverse transcriptase (InVitrogen) and PCR using oligonucleotide primers specific for HA, NA, and M1 proteins and Taq I DNA polymerase (InVitrogen) (Table 1). The PCR fragments were cloned into the bacterial subcloning vector, pCR2.1TOPO (InVitrogen), between Eco RI sites that resulted in three recombinant plasmids, containing the HA, NA, and M1 cDNA clones.

**Example 3**

**RT-PCR Cloning of Human Influenza A/Sydney/5/94 (H3N2) Viral Genes**

**[00207]** Influenza A/Sydney/5/97 (H3N2) Virus was obtained from Dr. M. Massare (Novavax, Inc., Rockville, Md.). Viral genomic RNA was isolated by the RNA acid phenol extraction method under BSL2 containment conditions at Novavax, Inc. using Trizol LS reagent (Invitrogen). cDNA molecules of the viral RNAs were obtained by reverse transcription and PCR using oligonucleotide primers specific for HA, NA, M1, M2, and NP proteins (Table 1). The PCR fragments were cloned into the bacterial subcloning vector, pCR2.1TOPO, between Eco RI sites that resulted in five recombinant plasmids, containing the HA, NA, M1, M2, and NP cDNA clones.

**Example 4**

**Cloning of Avian Influenza A/Hong Kong/1073/99 Viral cDNAs into Baculovirus Transfer Vectors**

**[00208]** From the pCR2.1TOPO-based plasmids, the HA, NA, or M1 genes were subcloned into pFastBac1 baculovirus transfer vector (InVitrogen) within the polyhedron locus and Tn7 att sites and downstream of the baculovirus polyhedrin promoter and upstream of the polyadenylation signal sequence. The viral genes were ligated with T4 DNA ligase. For the HA gene, a Bam HI-Kpn I DNA fragment from pCR2.1TOPO-HA was inserted into *BamH*I-*Kpn*I digested pFastBac1 plasmid DNA. For the NA gene, an *EcoR*I DNA fragment from pCR2.1TOPO-NA was inserted into *EcoR*I digested pFastBac1 plasmid DNA. For the M1 gene, an Eco RI DNA fragment from pCR2.1TOPO-M1 was inserted into Eco RI digested pFastBac1 plasmid DNA. Competent *E. coli* DH5α bacteria (InVitrogen) were transformed with these DNA ligation reactions, transformed colonies resulted, and bacterial clones isolated. The resulting pFastBac1-based plasmids, pFastBac1-HA, pFastBac1-NA, and pFastBac1-M1 were characterized by restriction enzyme mapping on agarose gels (FIG. 4A). The nucleotide sequences as shown on FIGS. 1-3 of the cloned genes were determined by automated DNA sequencing. DNA sequence analysis showed that the cloned influenza HA, NA, and M1 genes were identical to the nucleotide sequences for these genes as published previously [NA, HA, and M1 genes of influenza A/Hong Kong/1073/99 (H9N2) (GenBank accession numbers AJ404629, AJ404626, and AJ278646, respectively)].

**Example 5**

**Cloning of Human Influenza A/Sydney/5/97 Viral cDNAs into Baculovirus Transfer Vectors**

**[00209]** From the pCR2.1TOPO-based plasmids, the HA, NA, M1, M2, and NP genes were subcloned into pFastBac1 baculovirus transfer vector within the polyhedron locus and Tn7 att sites and downstream of the baculovirus polyhedrin promoter and upstream of the polyadenylation signal sequence. The viral genes were ligated with T4 DNA ligase. For the HA gene, a Bam HI-Kpn I DNA fragment from pCR2.1TOPO-hHA3 was inserted into *BamH*I-*Kpn*I digested pFastBac1 plasmid DNA. For the NA gene, an Eco RI DNA fragment from pCR2.1TOPO-hNA was inserted into *EcoR*I digested pFastBac1 plasmid DNA. For the M1 gene, an Eco RI DNA fragment from pCR2.1TOPO-hM1 was inserted into *EcoR*I digested pFastBac1 plasmid DNA. For the M2 gene, an *EcoR*I DNA fragment from pCR2.1TOPO-hM2 was inserted into *EcoR*I digested pFastBac1 plasmid DNA. For the NP gene, an *EcoR*I DNA fragment from pCR2.1TOPO-hNP was inserted into *EcoR*I digested pFastBac1 plasmid DNA. Competent *E. coli* DH5α bacteria were transformed with these DNA ligation reactions, transformed colonies resulted, and bacterial clones isolated. The resulting pFastBac 1-based plasmids, pFastBac1-hHA3, pFastBac1-hNA2, pFastBac1-hM1, pFASTBAC1-hM2, and pFASTBAC1-hNP were characterized by restriction enzyme mapping on agarose gels. The nucleotide sequences of the cloned genes were determined by automated DNA sequencing. DNA sequence analysis showed that the cloned influenza HA, NA, M1, M2, and NP genes were identical to the nucleotide sequences for these genes as published previously.

**Example 6**

**Construction of Multigenic Baculovirus Transfer Vectors Encoding Multiple Avian Influenza A/Hong Kong/1073/99 Viral Genes**

**[00210]** In order to construct pFastBac1-based bacmid transfer vectors expressing multiple influenza A/Hong Kong/1073/99 (H9N2) virus genes, initially a *Sna* BI-*Hpa* I DNA fragment from pFastBac1-M1 plasmid containing the M1 gene was cloned into Hpa I site of pFastBac1-HA. This resulted in pFastBac1-HAM plasmid encoding both HA and M1 genes within independent expression cassettes and expressed under the control of separate polyhedrin promoters.

[00211] Finally, a *SnaB*I-*Avr*II DNA fragment from pFastBac1-HAM containing the HA and M1 expression cassettes, was transferred into *Hpa* I-*Avr* II digested pFastBac1-NA plasmid DNA. This resulted in the plasmid pFastBac1-NAHAM encoding three independent expression cassettes for expression of influenza HA, NA, and M1 genes and expressed under the control of separate polyhedrin promoters (FIG. 4B).

[00212] In another example, the H3 gene from pFASTBAC1-hHA3 (see Example 5) was cloned into pFASTBAC1-NAHAM as a fourth influenza viral gene for the expression and production of heterotypic influenza VLPs.

**Example 7**

**Generation of Multigenic Recombinant Baculovirus Encoding NA, HA, and M1 Genes of Avian Influenza A/Hong Kong/1073/99 Virus in Insect Cells**

[00213] The resulting multigenic bacmid transfer vector pFastBac1-NAHAM was used to generate a multigenic recombinant baculovirus encoding avian influenza A/Hong Kong/1073/99 (H9N2) HA, NA, and M1 genes for expression in insect cells. Recombinant bacmid DNAs were produced by site-specific recombination at polyhedrin and Tn7 att DNA sequences between pFastBac1-NAHAM DNA and the AcMNPC baculovirus genome harbored in competent *E. coli* DH10BAC cells (InVitrogen) (FIG. 4B). Recombinant bacmid DNA was isolated by the mini-prep plasmid DNA method and transfected into Sf-9s cells using the cationic lipid CELLFECTIN (InVitrogen). Following transfection, recombinant baculoviruses were isolated, plaque purified, and amplified in Sf-9S insect cells. Virus stocks were prepared in Sf-9S insect cells and characterized for expression of avian influenza viral HA, NA, and M1 gene products. The resulting recombinant baculovirus was designated bNAHAM-H9N2.

**Example 8**

**Expression of Recombinant Avian Influenza A/Hong Kong/1073/99 Proteins in Insect Cells**

[00214] Sf-9S insect cells maintained as suspension cultures in shaker flasks at 28° C. in serum-free medium (HyQ SFM, HyClone, Ogden, Utah) were infected at a cell density of $2 \times 10^6$ cells/ml with the recombinant baculovirus, bNAHAM-H9N2, at a multiplicity of infection (MOI) of 3 pfu/cell. The virus infection proceeded for 72 hrs. to allow expression of influenza proteins. Expression of avian influenza A/Hong Kong/1073/99 (H9N2) HA and

M1 proteins in infected insect cells was confirmed by SDS-PAGE and Western immunoblot analyses. SDS-PAGE analysis was performed on 4-12% linear gradient NuPAGE gels (Invitrogen) under reduced and denaturing conditions. Primary antibodies in Western immunoblot analysis were polyclonal rabbit antiserum raised against avian influenza A/Hong Kong/1073/99 (H9N2) obtained from CDC and monoclonal murine antiserum to influenza M1 protein (Serotec, UK). Secondary antibodies for Western immunoblot analysis were alkaline phosphatase conjugated goat IgG antisera raised against rabbit or mouse IgG (H+L) (Kirkegaard and Perry Laboratories, Gaithersburg, Md., USA). Results of these analyses (FIG. 5) indicated that the HA and M1 proteins were expressed in the baculovirus-infected insect cells.

**Example 9**

**Purification of Recombinant Avian Influenza H9N2 Virus-Like Particles and Macromolecular Protein Complexes**

**[00215]** Culture supernatants (200 ml) from Sf-9S insect cells infected with the recombinant baculovirus bNAHAM-H9N2 that expressed avian influenza A/Hong Kong/1073/99 (H9N2) HA, NA, and M1 gene products were harvested by low speed centrifugation. Culture supernatants were clarified by centrifugation in a Sorval RC-5B superspeed centrifuge for 1 hr at 10,000 × g and 4° C using a GS-3 rotor. Virus and VLPs were isolated from clarified culture supernatants by centrifugation in a Sorval OTD-65 ultracentrifuge for 3 hr at 27,000 rpm and 4° C. using a Sorval TH-641 swinging bucket rotor. The virus pellet was resuspended in 1 ml of PBS (pH 7.2), loaded onto a 20-60% (w/v) discontinuous sucrose step gradient, and resolved by centrifugation in a Sorval OTD-65 ultracentrifuge for 16 hr at 27,000 rpm and 4° C using a Sorval TH-641 rotor. Fractions (0.5 ml) were collected from the top of the sucrose gradient.

**[00216]** Influenza proteins in the sucrose gradient fractions were analyzed by SDS-PAGE and Western immunoblot analyses as described above in Example 6. The HA and M1 proteins were found in the same sucrose gradient fractions (FIG. 6) as shown by Western blot analysis and suggested that the HA and M1 proteins were associated as macromolecular protein complexes. Also the HA and M1 proteins were found in fractions throughout the sucrose gradient suggesting that these recombinant viral proteins were associated with macromolecular protein complexes of different densities and compositions.

## Example 10

## Analysis of Recombinant Avian Influenza H9N2 VLPs and Proteins by Gel Filtration Chromatography

[00217] Protein macromolecules such as VLPs and monomeric proteins migrate differently on gel filtration or size exclusion chromatographic columns based on their mass size and shape. To determine whether the recombinant influenza proteins from sucrose gradient fractions were monomeric proteins or macromolecular protein complexes such as VLPs, a chromatography column (7 mm × 140 mm) with a resin bed volume of 14 ml of Sepharose CL-4B (Amersham) was prepared. The size exclusion column was equilibrated with PBS and calibrated with Dextran Blue 2000, Dextran Yellow, and Vitamin B12 (Amersham Pharmacia) with apparent molecular weights of 2,000,000; 20,000; and 1,357, respectively, to ascertain the column void volume. Dextran Blue 2000 eluted from the column in the void volume (6 ml fraction) also. As expected, the recombinant influenza protein complexes eluted from the column in the void volume (6 ml fraction). This result was characteristic of a high molecular weight macromolecular protein complex such as VLPs. Viral proteins in the column fractions were detected by Western immunoblot analysis as described above in Example 6. The M1 proteins were detected in the void volume fractions (FIG. 7). As expected baculovirus proteins were also in the void volume.

## Example 11

## Electron Microscopy of Recombinant Influenza VLPs

[00218] To determine whether the macromolecular protein complexes isolated on sucrose gradients and containing recombinant avian influenza proteins had morphologies similar to influenza virions, electron microscopic examination of negatively stained samples was performed. Recombinant avian influenza A/Hong Kong/1073/99 (H9N2) protein complexes were concentrated and purified from culture supernatants by ultracentrifugation on discontinuous sucrose gradients as described in Example 7. Aliquots of the sucrose gradient fractions were treated with a 2% glutaraldehyde in PBS, pH7.2, absorbed onto fresh discharged plastic/carbon-coated grids, and washed with distilled water. The samples were stained with 2% sodium phosphotungstate, pH 6.5, and observed using a transmission electron microscope (Philips). Electron micrographs of negatively-stained samples of recombinant avian influenza H9N2 protein complexes from two sucrose gradient fractions showed spherical and rod-shaped particles (FIG. 8) from two sucrose gradient fractions. The

particles had different sizes (60 and 80 nm) and morphologies. Larger complexes of both types of particles were also detected, as well as rod-shaped particles (FIG. 8). All observed protein complex structures exhibited spike like surface projections resembling influenza virus HA and NA peplomers. Since the size and appearance of the 80 nm particles was similar to that of wild type influenza virus particles, these structures likely represented enveloped influenza VLPs. The smaller particles of approximately 60 nm probably represented subviral particles that differed from the above VLPs both morphologically and structurally.

**Example 12**

**Analysis of Functional Characteristics of Influenza Proteins by Hemagglutination Assay**

**[00219]** To determine whether the purified influenza VLPs and proteins possessed functional activities, such as hemagglutination and neuraminidase activity, which were characteristic for influenza virus, the purified influenza VLPs and proteins were tested in hemagglutination and neuraminidase assays.

**[00220]** For the hemagglutination assay, a series of 2-fold dilutions of sucrose gradient fractions containing influenza VLPs or positive control wild type influenza virus type A were prepared. Then they were mixed with 0.6% guinea pig red blood cells in PBS (pH 7.2) and incubated at 4° C. for 1 to 16 hr. As a negative control, PBS was used. The extent of hemagglutination was determined visually, and the highest dilution of fraction capable of agglutinating guinea pig red blood cells was determined (FIG. 9). The highest hemagglutination titer observed for the purified influenza VLPs and proteins was 1:4000, which was higher than the titer shown by the wild type influenza control, which was 1:2000.

**Example 13**

**Analysis of Functional Characteristics of Influenza Proteins by Neuraminidase Assay**

**[00221]** The amount of neuraminidase activity in influenza VLP-containing sucrose gradient fractions was determined by the neuraminidase assay. In this assay the NA (an enzyme) acted on the substrate (fetuin) and released sialic acid. Arsenite reagent was added to stop enzyme activity. The amount of sialic acid liberated was determined chemically with the thiobarbituric acid that produced a pink color in proportion to free sialic acid. The amount of color (chromophor) was measured in a spectrophotometer at wavelength 594 nm. The data, as depicted in FIG. 8, showed that a significant amount of sialic acid was produced by VLP-containing fractions of the sucrose gradients and that these fractions corresponded to those fractions exhibiting hemagglutination activity.

**Example 14**

**Immunization of BALB/c Mice with Functional Homotypic Recombinant Influenza H9N2 VLPs**

**[00222]** The immunogenicity of the recombinant influenza VLPs was ascertained by immunization of mice followed by Western blot analysis of immune sera. Recombinant VLPs (1 μg/injection) comprised of viral HA, NA, and M1 proteins from avian influenza virus type A/Honk Kong/1073/99 and purified on sucrose gradients were inoculated subcutaneously into the deltoid region of ten (10) female BALB/c mice at day 0 and day 28 (FIG. 11). PBS (pH 7.2) was administered similarly as a negative control into five (5) mice. The mice were bled from the supraorbital cavity at day-1 (pre-bleed), day 27 (primary bleed), and day 54 (secondary bleed). Sera were collected from blood samples following overnight clotting and centrifugation.

**[00223]** For Western blot analysis, 200 ng of inactivated avian influenza virus type A H9N2 or cold-adapted avian influenza virus type A H9N2, as well as See Blue Plus 2 pre-stained protein standards (InVitrogen), was denatured (95° C., 5 minutes) and subjected to electrophoresis under reduced conditions (10 mM β-mercaptoethanol) on 4-12% polyacrylamide gradient NuPAGE gels (InVitrogen) in MES buffer at 172 volts until the bromophenol blue tracking dye disappeared. For protein gels, the electrophoreses proteins were visualized by staining with Colloidal Coomassie Blue reagent (InVitrogen). Proteins were transferred from the gel to nitrocellulose membranes in methanol by the standard Western blot procedure. Sera from VLP-immunized mice and rabbits immunized with inactivated avian influenza virus H9N2 (positive control sera) were diluted 1:25 and 1:100, respectively, in PBS solution (pH 7.2) and used as primary antibody. Protein bound membranes, which were blocked with 5% casein, were reacted with primary antisera for 60 minutes at room temperature with constant shaking. Following washing of primary antibody membranes with phosphate buffered saline solution containing Tween 20, secondary antisera [goat anti-murine IgG--alkaline phosphatase conjugate (1:10,000) or goat anti-rabbit IgG --alkaline phosphatase conjugate (1:10,000)] were reacted 60 minutes with the membrane. Following washing of secondary antibody membranes with phosphate buffered saline solution containing Tween 20, antibody-binding proteins on the membranes were visualized by development with the chromogenic substrate such as NBT/BCIP (InVitrogen).

**[00224]** The results of Western blot analysis (FIG. 12) were that proteins with molecular weights similar to viral HA and M1 proteins (75 and 30 kd, respectively) bound to positive control sera (FIG. 12B) and sera from mice immunized with the recombinant influenza H9N2 VLPs (FIG. 12A). These results indicated that the recombinant influenza H9N2 VLPs alone were immunogenic in mice by this route of administration.

**Example 15**

**Kong/1073/99 (H9N2) VLP Immunogenicity And Challenge Study In BALB/c Mice**

**[00225]** BALB/C mice were immunized with H9N2 VLPs (1 μg HA or 10 μg HA/dose), with or without 100 μg Novasome adjuvant, on day 0 and day 21 and challenged with homologous infectious virus IN on day 57. Mice were bled on days 0, 27 and 57 with the serum assayed for anti-HA antibodies by the hemagglutination inhibition assay (HI) using turkey RBCs, and influenza by ELISA. Results of this study are shown in Figure 13 through Figure 16.

**[00226]** High titers of H9N2 antibodies were induced after a single immunization (primary) with H9N2 VLP vaccine without or with Novasomes and a dose of 10 μg VLPs containing 1 μg HA (Figure 13). Specific antibody titers were increased about half to one log following a booster immunization.

**[00227]** After immunization and a boost with 1 μg of HA in the form of H9N2 VLPs the serum HI levels were at or above the level generally considered protective (log2 = 5) in all animals (Figure 14, lower left panel). H9N2 VLPs formulated with Novasome adjuvant increased HI responses about 2 fold following primary immunization and about 4 fold after the booster (Figure 14, lower right panel). Purified subunit H9N2 hemagglutinin also induced protective levels of HI antibodies after boosting and Novasomes again increased HI antibody responses by about 2 fold after the primary and 4 fold after the booster immunizations (Figure 14, upper panels). The level of HI antibody induced with 10 μg of HA given as a subunit vaccine was equivalent to 1 μg of HA presented in the form of a VLP.

**[00228]** In addition, weight loss was significantly less in the mice immunized with H9N2 VLPs or with VLPs plus adjuvant compared to unvaccinated control animals (Figure 15). There was no statistical difference in weight loss in the groups immunized with H9N2 VLPs and H9N2 VLPs plus Novasome adjuvant.

[00229] Likewise, lung virus titers at 3 and 5 days post challenge with H9N2 virus were significantly reduced in mice immunized with H9N2 VLPs (Figure 16). At day 3 when the influenza virus titers peak in the lung tissues, mice immunized with H9N2 VLPs plus Novasomes® had a significantly greater reduction in virus titer compared to mice immunized with VLPs alone and the unvaccinated control mice.

**Example 16**

**A/Fujian/411/2002 (H3N2) VLP Immunogenicity and Cross Reactivity between several influenza Strains**

[00230] BALB/c mice were immunized with A/Fujian/411/2002 VLPs (3.0, 0.6, 0.12 and 0.24μg HA/dose), twice IM and IN. Mice were bled on days 0 and 35. The serum was then assayed for anti-HA antibodies by the hemagglutination inhibition assay (HI) using turkey RBCs, and for anti-influenza antibodies by ELISA. Results of this study are shown on Figures 17A, 17B and 17C. These results indicate that an immune response was mounted both IM and IN against HA and NA.

**Example 17**

**Determination of the IgG isotypes in mouse after inoculation with H3N2 VLPs**

[00231] Mice were inoculated with VLPs intramuscularly and intranasal. At week 5 sera was collected and assayed to distinguish between IgG isotypes.

[00232] Sera was tested on plates coated with purified HA (Protein Sciences) A/Wyoming/3/2003 using an ELISA assay. Serial five-fold dilutions of sera was added to the wells and the plates were incubated. Next, the biotinylated goat anti-mouse Ig, or anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b and anti-mouse IgG3. Then, streptavidine-peroxidase was added to the wells. Bound conjugates were detected. Results are illustrated on Figures 18A and B. These results illustrate that IgG2a are the most abundant isotype in an immune response against VLPs in mouse.

**Example 18**

**A/Hong Kong/1073/99 (H9N2) VLP dose-ranging study in SD rats**

[00233] SD rats (n=6 per dose) were immunized on day 0 and day 21 with purified A/Hong Kong/1073/99 (H9N2) VLPs diluted with PBS at neutral pH to 0.12, 0.6, 3.0, and 15.0 μg

HA or with PBS alone. Blood samples were taken from the animals on day 0, day 21, day 35 and day 49 and the serum assayed for hemagglutination inhibition assay (HI) to detect functional antibodies able to inhibit the binding function of the HA. The dosage was based on HA content as measured using SDS-PAGE and scanning densitometry of purified H9N2 VLPs. Hemagglutinin inhibition assay titer results are depicted in Figure 19. A single 0.6 μg HA dose of H9N2 VLPs or two doses of 0.12 μg HA produced protective levels of HI antibodies in rats. These data indicate that a lower amount of HA can induce a protective response when said HA is part of a VLP.

**Example 19**

**Kong/1073/99 (H9N2) VLP Immunogenicity**

**[00234]** BALB/C mice were immunized with H9N2 VLPs (0.12, 0.6 μg HA /dose), with or without 100 μg Novasome and Alum adjuvant, on day 0 and day 21 and challenged with homologous infectious virus IN on day 57. Mice were also immunized with 3.0 and 15.0 μg HA /dose (no adjuvant). Mice were bled on days 0, 21, 35 and 49 with the serum assayed for anti-HA antibodies by the hemagglutination inhibition assay (HI) using turkey RBCs, and influenza by ELISA. Results of this study are shown in Figures 20 A and B.

**[00235]** The results indicate that a more robust overall immune response was observed when the VLPs were administered with an adjuvant. However, a protective response was elicited with 0.12 μg HA /dose at week 3 when compared to the VLPs formulation with Alum and VLPs with no adjuvant. Also in week 7, the VLPs comprising Novasomes had about 2 log increase in HI titer as compared to the VLP with Alum. The robustness of the response was similar to VLPs administered at 3.0 and 15.0 μg HA /dose without an adjuvant. These results indicate that Novasomes elicit a more robust response as compared to Alum. In addition, a protective immune response can be achieved with 25× less VLPs when said VLPs are administered in a formulation comprising Novasomes.

**[00236]** Also, in the 0.6 μg HA /dose data, the Novasome formulation had an about 1.5 log greater response than compared to Alum. The immune responses were similar in magnitude to VLPs administered in 3.0 and 15.0 μg HA /dose without adjuvant. These results indicate that with an adjuvant, approximately 5× less VLPs are needed to be administered to achieve a protective response.

**[00237]** Also, Figure 20B depicts the HI titer of H9N2 VLPs using different formulations of Novasomes. The following are the formulas used in the experiment:

Group 1: H9N2 VLP (0.1μg) (n=5)
Group 2: H9N2 VLP (0.1μg) w/ DCW neat) (n=5)
Group 3: H9N2 VLP (0.1μg) w/ DCW 1:3) (n=5)
Group 4: H9N2 VLP (0.1μg) w/ DCW 1:9) (n=5)
Group 5: H9N2 VLP (0.1μg) w/ DCW 1:27) (n=5)
Group 6: H9N2 VLP (0.1μg) w/ NVAX 1) (n=5)
Group 7: H9N2 VLP (0.1μg) w/ NVAX 2) (n=5)
Group 8: H9N2 VLP (0.1μg) w/ NVAX 3) (n=5)
Group 9: H9N2 VLP (0.1μg) w/ NVAX 4) (n=5)
Group 10: H9N2 VLP (0.1μg) w/ NVAX 5) (n=5)
Group 11: H9N2 VLP (0.1μg) w/ Alum-OH) (n=5)
Group 12: H9N2 VLP (0.1μg) w/ CpG) (n=5)
Group 13: PBS (0.6μg) (n=5)
Group 14: H3 VLPs (0.6μg) (n=5)
Group 15: H5 VLPs (0.6μg) (n=8)

-H9: (Lot# 11005)
-DCW: Novasomes (Lot#121505-2, Polyoxyethylene-2-cetyl ether, Cholesterol, Superfined soybean oil, and Cetylpridinium chloride)
-NVAX 1: B35P83, MF-59 replica (Squalene, Polysorbate, and Span)
-NVAX 2: B35P87 (Soybean Oil, Brij, Cholesterol, Pluronic F-68)
-NVAX 3: B35P88 (Soybean Oil, Brij, Cholesterol, Pluronic F-68, and Polyethyleneimine)

-NVAX 4: B31P60 (Squalene, Brij, Cholesterol, Oleic acid)
-NVAX 5: B31P63 (Soybean oil, Glyceryl monostearate, Cholesterol, Polysorbate)
-CpG: (Lot# 1026004)
-H5: (Lot# 22406)

**[00238]** Figure 21 depicts and H9N2 VLP dose response curve. This data indicates that a dose of VLPs at 0.6 μg HA /dose is the minimum to elicit a protective immune response in mice after 3 weeks.

**Example 20**

**Materials and Methods for Ferret Studies**

**[00239]** Ferrets were purchased from Triple F Farms (FFF, Sayre, PA). All ferrets purchased has an HAI titer of less that 10 hemagglutination units. Approximately two days prior to vaccination, animals were implanted with a temperature transponder (BioMedic Data Systems, Inc.). Animal (6 animals/group) were vaccinated on day 0 either with (1) PBS (negative control, group one), (2) H3N2 influenza VLPs @ 15 μg of H3 (group 2), (3) H3N2 influenza VLPs @ 3 μg of H3 (group 2), (4) H3N2 influenza VLPs @ 0.6 μg of H3 (group 3), (5) H3N2 influenza VLPs @ 0.12 μg of H3 (group 5), or (6) rH3HA @ 15 μg (group 6).

On day 21 animals were boosted with vaccine. Animals were bled on days 0 (prior to vaccination), day 21 (prior to vaccine boost), and day 42. Animals were assessed for clinical signs of adverse vaccine effects once weekly throughout the study period. Similar studies were performed with other influenza VLPs.

**HAI levels in ferret sera**

**[00240]** Ferret sera were obtained from FFF, treated with Receptor Destroying Enzyme (RDE) and tested in a hemagglutination inhibition (HAI) assay by standard procedures (Kendal *et al.* (1982)). All ferrets that were chosen for the study tested negative (HAI =10) for pre-existing antibodies to currently circulating human influenza virus (A/New Caledonia/20/99 (H1N1), A panama/2007/99 (H3N2), A/Wellington/01/04 (H2N3) and B/Sichuan/379/99 and H5N1).

**Ferrets**

**[00241]** Approximately 8 month-old, influenza naïve, castrated and descented, male Fitch ferrets (*Mustela putorius furo*) were purchased form FFF. Animals were housed in stainless steel rabbit cages (Shor-line, KS) containing Sani-chips Laboratory Animal Bedding (P.J. Murphy Forest Products, NJ). Ferrets were provided with Teklad Global Ferret Diet (Harlan Teklad, WI) and fresh water *ad libitum*. Pans were changed three times each week, and cages were cleaned biweekly.

**Vaccinations and Blood Collection of Ferrets**

**[00242]** The vaccine, H3N2 influenza VLPs or H9N2 influenza VLPs and controls, for example, rH3NA (A/Wyoming/3/2003) and PBS (negative control) were stored at 4° C prior to use. For most studies, six groups of ferrets (N-6/group) were vaccinated intramuscularly with either concentration of vaccine or control in a volume of 0.5 ml.

**[00243]** Prior to blood collection and vaccination, animals were anesthetized by intramuscular injection into the inner thigh with a solution of Katamine (25 mg/kg, Atropine (0.05 mg/kg) and Xylazine (2.0 mg/kg) "KAX." Once under anesthesia, ferrets were positioned in dorsal recumbency and blood was collected (volume between 0.5 and 1.0 ml) from the anterior vena cava using a 23 gauge 1'' needle connected to a 1 cc tuberculin syringe. Blood was transferred to a tube containing a serum separator and clot activator and allowed to clot at room temperature. Tubes were centrifuged and sera was removed and frozen at -80° C. Blood was collected prior to vaccination (day 0), prior to boost (day 21) and day 42 and tested by HAI assay.

**Monitoring of Ferrets**

**[00244]** Temperatures were measured weekly at approximately the same time throughout the study period. Pre-vaccination values were averaged to obtain a baseline temperature for each ferret. The change in temperature (in degrees Fahrenheit) was calculated at each time point for each animal. Ferrets were examined weekly for clinical signs of adverse vaccine effects, including temperature, weight loss, loss of activity, nasal discharge, sneezing and diarrhea. A scoring system bases on that described by Reuman *et al.* (1989) was used to assess activity level where 0 = alert and playful; 1 = alert but playful only when stimulated; 2 = alert by not playful when stimulated; 3 = neither alert not playful when stimulated. Based on the scores for each animal in a group, a relative inactivity index was calculated as ∑(day0 –Day 42)[activity score +1]/ ∑(day0 –Day 42), where n equals the total number of observations. A value of 1 was added to each base score so that a score of "0" could be divided by a denominator, resulting in an index value of 1.0.

**Serum Preparations**

**[00245]** Sera generally have low levels of non-specific inhibitors on hemagglutination. To inactivate these non-specific inhibitors, sera were treated with (RDE) prior to being tested. Briefly, three part RDE was added to one part sera and incubated overnight at 37° C. RDE was inactivated by incubation at 56° C for approximately 30 minutes. Following inactivation of RDE, PBS was added to the sample for a final serum dilution of 1:10 (RDE-Tx). The diluted RDE-Tx sera was stored at 4° C prior to testing (for 7 days) or stored at -20° C.

**Preparation turkey erythrocytes:**

**[00246]** Human influenza viruses bind to sialic acid receptors containing N-acetylneuraminic acid α 2,6-galactose linkages. Avian influenza viruses bind to sialic acid receptors containing N-acetylneuraminic acid α 2,3 galactose (α 2,3 linkages) and express both α 2,3 and α 2,6 linkages. Turkey erythocytes (TRBC) are used for the HAI assay since A/Fujian is a human influenza virus. The TRBCs adjusted with PBS to achieve a 0.5% vol/vol suspension. The cells are kept at 4 °C and used within 72 hours of preparation.

**HAI Assay**

**[00247]** The HAI assay was adapted from the CDC laboratory-based influenza surveillance manual (Kendal *et al.* (1982) Concepts and procedures for laboratory based influenza surveillance, U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Atlanta, Georgia, herein incorporated by reference in its entirety for all purposes). RDE-Tx sera was serially two-fold diluted in v-bottom microtiter plates. An equal volume of virus adjusted, adjusted to approximately 8 HAU/50ul was added to each well. The plates were covered and incubated at room temperature for 15 minutes followed by the addition of 0.5% TRBC. The plates were mixed by agitation, covered, and the TRBC were allowed to settle for 30 minutes at room temperature. The HAI titer was determined by the reciprocal dilution of the last row which contained non-agglutinated TRBC. Positive and negative serum controls were included for each plate.

**Example 21**

**A/Hong Kong/1073/99 (H9N2) VLP dose-ranging study in Ferrets**

**[00248]** Ferrets, serologically negative by hemagglutination inhibition for influenza viruses, were used to assess the antibody and HI titer after an inoculation with H9N2 VLPs. Ferrets were bled on days 0, and 21 days with the serum assayed for anti-HA antibodies by the hemagglutination inhibition assay (HI) using turkey RBCs, and for anti-influenza antibodies by ELISA. Results are illustrated in Figure 22. These results show HI titers corresponding to protective antibody levels at VLP doses of 1.5 and 15 µg.

**Example 21**

**Vaccination of H3N2 VLPs in Ferrets**

**[00249]** Ferrets were vaccinated at day 0, and given a boost on day 21 with different strains of H3N2 VLPs at different dosages (HA dosages of 0.12, 0.6, 3.0, 15.0 µg). The positive control was rH3HA at 15 µg and PBS alone is the negative control. Sera, as described above, were taken from the ferrets on day 0 prior to vaccination, day 21 (prior to boost) and day 42. An HI assay was conducted on the serum samples to determine if there was an immune response against the VLPs. These data are illustration on Figure 23. These data indicate that H3N2 VLPs, when introduced into ferrets, do induce an immune response. Thus, the H3N2 VLPs are immunogenic in ferrets.

**Example 22**

**RT-PCR and cloning of HA, NA, and M1 genes of influenza A/Indonesia/5/05 (H5N1) virus**

**[00250]** Clade 2 influenza virus, strain A/Indonesia/5/05 (H5N1) viral RNA was extracted using Trizol LS (Invitrogen, Carlsbad, CA) under BSL-3 containment conditions. Reverse transcription (RT) and PCR were performed on extracted viral RNA using the One-Step RT-PCR system (Invitrogen) with gene-specific oligonucleotide primers. The following primer pairs were used for the synthesis of the H5N1 hemagglutinin (HA), neuraminidase (NA), and matrix (M1) genes, respectively:

5'-AACGGTCCGATGGAGAAAATAGTGCTTCTTC-3' (SEQ ID. 4) and
5'-AAAGCTTTTAAATGCAAATTCTGCATTGTAACG-3' (SEQ ID. 5) (HA);
5'-AACGGTCCGATGAATCCAAATCAGAAGATAAT-3' (SEQ ID. 6)and
5'-AAAGCTTCTACTTGTCAATGGTGAATGGCAAC-3' (SEQ ID. 7) (NA); and
5'-AACGGTCCGATGAGTCTTCTAACCGAGGTC-3' (SEQ ID. 8)and
5'-AAAGCTTTCACTTGAATCGCTGCATCTGCAC-3' (SEQ ID. 9) (M1) (ATG codons are underlined).

**[00251]** Following RT-PCR, cDNA fragments containing influenza HA, NA, and M1 genes with molecular weights of 1.7, 1.4, and 0.7 kB, respectively, were cloned into the pCR2.1-TOPO vector (Invitrogen). The nucleotide sequences of the HA, NA, and M1 genes were determined by DNA sequencing. A similar strategy was followed for cloning a clade 1 H5N1 influenza virus from Vietnam/1203/2003.

**Example 23**

**Generation of recombinant baculoviruses comprising H5N1**

**[00252]** The HA gene was cloned as a *RsrII-HindIII* DNA fragment (1.7 kb) downstream of the *Ac*MNPV polyhedrin promoter within pFastBac1 bacmid transfer vector (Invitrogen) digested with *RsrII and HindIII*. Similarly, the NA and M1 genes were cloned as *Eco*RI-*HindIII* DNA fragments (1.4 and 0.8 kb, respectively) into *Eco*RI-HindIII-digested pFastBac1 plasmid DNA. The three resulting baculovirus transfer plasmids pHA, pNA, and pM1 containing influenza A/Indonesia/5/05 (H5N1)virus HA, NA, and M1 genes, respectively, were used to generate recombinant bacmids.

[00253] Bacmids were produced by site-specific homologous recombination following transformation of bacmid transfer plasmids containing influenza genes into *E. coli* DH10Bac competent cells, which contained the *Ac*MNPV baculovirus genome (Invitrogen). The recombinant bacmid DNA was transfected into the Sf9 insect cells.

Nucleotide sequences of the Indonesia/5/05 HA, NA, and M1 genes.

HA (SEQ ID. 10)

```
ATGGAGAAAATAGTGCTTCTTCTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC
ATTGGTTACCATGCAAACAATTCAACAGAGCAGGTTGACACAATCATGGAAAAGAACGTT
ACTGTTACACATGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTA
GATGGAGTGAAGCCTCTAATTTTAAGAGATTGTAGTGTAGCTGGATGGCTCCTCGGGAAC
CCAATGTGTGACGAATTCATCAATGTACCGGAATGGTCTTACATAGTGGAGAAGGCCAAT
CCAACCAATGACCTCTGTTACCCAGGGAGTTTCAACGACTATGAAGAACTGAAACACCTA
TTGAGCAGAATAAACCATTTTGAGAAAATTCAAATCATCCCCAAAAGTTCTTGGTCCGAT
CATGAAGCCTCATCAGGAGTGAGCTCAGCATGTCCATACCTGGGAAGTCCCTCCTTTTTT
AGAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAGAAAAGCTAC
AATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGAATTCACCATCCTAATGATGCG
GCAGAGCAGACAAGGCTATATCAAAACCCAACCACCTATATTTCCATTGGGACATCAACA
CTAAACCAGAGATTGGTACCAAAAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGA
AGGATGGAGTTCTTCTGGACAATTTTAAAACCTAATGATGCAATCAACTTCGAGAGTAAT
GGAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGACTCAGCAATT
ATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCG
ATAAACTCTAGTATGCCATTCCACAACATACACCCTCTCACCATCGGGGAATGCCCCAAA
TATGTGAAATCAAACAGATTAGTCCTTGCAACAGGGCTCAGAAATAGCCCTCAAAGAGAG
AGCAGAAGAAAAAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGG
CAGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAGTGGGTAC
GCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCA
ATCATTGACAAAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAATAACTTAGAA
AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTTCTAGATGTCTGGACTTAT
AATGCCGAACTTCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAAT
GTTAAGAACCTCTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAGGAGCTGGGT
AACGGTTGTTTCGAGTTCTATCACAAATGTGATAATGAATGTATGGAAAGTATAAGAAAC
GGAACGTACAACTATCCGCAGTATTCAGAAGAAGCAAGATTAAAAAGAGAGGAAATAAGT
GGGGTAAAATTGGAATCAATAGGAACTTACCAAATACTGTCAATTTATTCAACAGTGGCG
AGTTCCCTAGCACTGGCAATCATGATGGCTGGTCTATCTTTATGGATGTGCTCCAATGGA
TCGTTACAATGCAGAATTTGCATTTAA
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NA (SEQ ID. 11)

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ATGAATCCAAATCAGAAGATAATAACCATTGGATCAATCTGTATGGTAATTGGAATAGTT
AGCTTAATGTTACAAATTGGGAACATGATCTCAATATGGGTCAGTCATTCAATTCAGACA
GGGAATCAACACCAAGCTGAATCAATCAGCAATACTAACCCTCTTACTGAGAAAGCTGTG
GCTTCAGTAACATTAGCGGGCAATTCATCTCTTTGCCCCATTAGAGGATGGGCTGTACAC
AGTAAGGACAACAATATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATTAGAGAGCCG
TTCATCTCATGCTCCCACCTGGAATGCAGAACTTTCTTCTTGACTCAGGGAGCCTTGCTG
AATGACAAGCACTCCAACGGGACTGTCAAAGACAGAAGCCCTCACAGAACATTAATGAGT
TGTCCTGTGGGTGAGGCTCCCTCTCCATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCA
GCAAGTGCTTGCCATGATGGCACCAGTTGGTTGACAATTGGAATTTCTGGCCCAGACAAT
GAGGCTGTGGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTTGGAGG
AACAACATACTGAGAACTCAAGAGTCTGAATGTGCATGTGTAAATGGCTCTTGCTTTACT
GTAATGACTGATGGACCAAGTGATGGGCAGGCATCATATAAGATCTTCAAAATGGAAAAA
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GGAAAAGTGGTCAAATCAGTCGAATTGGATGCTCCTAATTATCACTATGAGGAATGCTCC
TGTTATCCTGATGCCGGCGAAATCACATGTGTTTGCAGGGATAATTGGCATGGCTCAAAT
AGGCCATGGGTATCTTTCAATCAAAATTTGGAGTATCAAATAGGATATATATGCAGTGGA
GTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGTAGTTGTGGCCCGATGTCCCCT
AACGGGGCATATGGGGTAAAAGGGTTTTCATTTAAATACGGCAATGGTGTTTGGATCGGG
AGAACCAAAAGCACTAATTCCAGGAGCGGCTTTGAAATGATTTGGGATCCAAATGGGTGG
ACTGGAACGGACAGTAGCTTTTCAGTGAAACAAGATATAGTAGCAATAACTGATTGGTCA
GGATATAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGATTAGATTGCATAAGACCT
TGTTTCTGGGTTGAGTTAATCAGAGGGCGGCCCAAAGAGAGCACAATTTGGACTAGTGGG
AGCAGCATATCTTTTTGTGGTGTAAATAGTGACACTGTGAGTTGGTCTTGGCCAGACGGT
GCTGAGTTGCCATTCACCATTGACAAGTAG
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M1 (SEQ ID. 12)

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ATGAGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATCCCGTCAGGCCCCCTC
AAAGCCGAGATCGCGCAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGATCTCGAG
GCTCTCATGGAGTGGCTGAAGACAAGACCAATCCTGTCACCTCTGACTAAAGGGATTTTG
GGATTTGTATTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGTC
CAGAATGCCCTAAATGGAAATGGAGATCCAAATAATATGGATAGGGCAGTTAAGCTATAT
AAGAAGCTGAAAAGAGAAATAACATTCCATGGGGCTAAAGAGGTTTCACTCAGCTACTCA
ACCGGTGCACTTGCCAGTTGCATGGGTCTCATATACAACAGGATGGGAACGGTGACTACG
GAAGTGGCTTTTGGCCTAGTGTGTGCCACTTGTGAGCAGATTGCAGATTCACAGCATCGG
TCTCACAGGCAGATGGCAACTATCACCAACCCACTAATCAGGCATGAAAACAGAATGGTG
CTGGCCAGCACTACAGCTAAGGCTATGGAGCAGATGGCGGGATCAAGTGAGCAGGCAGCG
GAAGCCATGGAGGTCGCTAATCAGGCTAGGCAGATGGTGCAGGCAATGAGGACAATTGGA
ACTCATCCTAACTCTAGTGCTGGTCTGAGAGATAATCTTCTTGAAAATTTGCAGGCCTAC
CAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTGA
```

[00254] One cloned HA gene, pHA5, contained two nucleotide changes, nt #1172 and nt #1508 (in the wt), as compared to the wild-type HA gene sequence. A similar strategy was followed for constructing and creating clade 1 H5N1 influenza virus from Vietnam/1203/2003 VLPs (see below). The alignments of pHA5 nucleotide and amino acid sequences follow.

```
wt      1 ...................ATGGAGAAAATAGTGCTTCTTCTTGCAATAG 31   seq id 10
                             |||||||||||||||||||||||||||||||
pHA5   51 ATTCGCCCTTAACGGTCCGATGGAGAAAATAGTGCTTCTTCTTGCAATAG 100  seq id 56
                   .         .         .         .         .
       32 TCAGTCTTGTTAAAAGTGATCAGATTTGCATTGGTTACCATGCAAACAAT 81
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      101 TCAGTCTTGTTAAAAGTGATCAGATTTGCATTGGTTACCATGCAAACAAT 150
                   .         .         .         .         .
       82 TCAACAGAGCAGGTTGACACAATCATGGAAAAGAACGTTACTGTTACACA 131
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      151 TCAACAGAGCAGGTTGACACAATCATGGAAAAGAACGTTACTGTTACACA 200
                   .         .         .         .         .
      132 TGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAG 181
          ||||||||||||||||||||||||||||||||||||||||||||||||||
      201 TGCCCAAGACATACTGGAAAAGACACACAACGGGAAGCTCTGCGATCTAG 250
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182 ATGGAGTGAAGCCTCTAATTTTAAGAGATTGTAGTGTAGCTGGATGGCTC 231
    ||||||||||||||||||||||||||||||||||||||||||||||||||
251 ATGGAGTGAAGCCTCTAATTTTAAGAGATTGTAGTGTAGCTGGATGGCTC 300

232 CTCGGGAACCCAATGTGTGACGAATTCATCAATGTACCGGAATGGTCTTA 281
    ||||||||||||||||||||||||||||||||||||||||||||||||||
301 CTCGGGAACCCAATGTGTGACGAATTCATCAATGTACCGGAATGGTCTTA 350

282 CATAGTGGAGAAGGCCAATCCAACCAATGACCTCTGTTACCCAGGGAGTT 331
    ||||||||||||||||||||||||||||||||||||||||||||||||||
351 CATAGTGGAGAAGGCCAATCCAACCAATGACCTCTGTTACCCAGGGAGTT 400

332 TCAACGACTATGAAGAACTGAAACACCTATTGAGCAGAATAAACCATTTT 381
    ||||||||||||||||||||||||||||||||||||||||||||||||||
401 TCAACGACTATGAAGAACTGAAACACCTATTGAGCAGAATAAACCATTTT 450

382 GAGAAAATTCAAATCATCCCCAAAAGTTCTTGGTCCGATCATGAAGCCTC 431
    ||||||||||||||||||||||||||||||||||||||||||||||||||
451 GAGAAAATTCAAATCATCCCCAAAAGTTCTTGGTCCGATCATGAAGCCTC 500

432 ATCAGGAGTGAGCTCAGCATGTCCATACCTGGGAAGTCCCTCCTTTTTTA 481
    ||||||||||||||||||||||||||||||||||||||||||||||||||
501 ATCAGGAGTGAGCTCAGCATGTCCATACCTGGGAAGTCCCTCCTTTTTTA 550

482 GAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAG 531
    ||||||||||||||||||||||||||||||||||||||||||||||||||
551 GAAATGTGGTATGGCTTATCAAAAAGAACAGTACATACCCAACAATAAAG 600

532 AAAAGCTACAATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGAAT 581
    ||||||||||||||||||||||||||||||||||||||||||||||||||
601 AAAAGCTACAATAATACCAACCAAGAAGATCTTTTGGTACTGTGGGGAAT 650

582 TCACCATCCTAATGATGCGGCAGAGCAGACAAGGCTATATCAAAACCCAA 631
    ||||||||||||||||||||||||||||||||||||||||||||||||||
651 TCACCATCCTAATGATGCGGCAGAGCAGACAAGGCTATATCAAAACCCAA 700

632 CCACCTATATTTCCATTGGGACATCAACACTAAACCAGAGATTGGTACCA 681
    ||||||||||||||||||||||||||||||||||||||||||||||||||
701 CCACCTATATTTCCATTGGGACATCAACACTAAACCAGAGATTGGTACCA 750

682 AAAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGAGTT 731
    ||||||||||||||||||||||||||||||||||||||||||||||||||
751 AAAATAGCTACTAGATCCAAAGTAAACGGGCAAAGTGGAAGGATGGAGTT 800

732 CTTCTGGACAATTTTAAAACCTAATGATGCAATCAACTTCGAGAGTAATG 781
    ||||||||||||||||||||||||||||||||||||||||||||||||||
801 CTTCTGGACAATTTTAAAACCTAATGATGCAATCAACTTCGAGAGTAATG 850

782 GAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGAC 831
    ||||||||||||||||||||||||||||||||||||||||||||||||||
851 GAAATTTCATTGCTCCAGAATATGCATACAAAATTGTCAAGAAAGGGGAC 900

832 TCAGCAATTATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTG 881
    ||||||||||||||||||||||||||||||||||||||||||||||||||
901 TCAGCAATTATGAAAAGTGAATTGGAATATGGTAACTGCAACACCAAGTG 950

882 TCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCATTCCACAACATAC 931
    ||||||||||||||||||||||||||||||||||||||||||||||||||
951 TCAAACTCCAATGGGGGCGATAAACTCTAGTATGCCATTCCACAACATAC 1000
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              .         .         .         .         .
 932 ACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTA 981
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1001 ACCCTCTCACCATCGGGGAATGCCCCAAATATGTGAAATCAAACAGATTA 1050
              .         .         .         .         .
 982 GTCCTTGCAACAGGGCTCAGAAATAGCCCTCAAAGAGAGAGCAGAAGAAA 1031
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1051 GTCCTTGCAACAGGGCTCAGAAATAGCCCTCAAAGAGAGAGCAGAAGAAA 1100
              .         .         .         .         .
1032 AAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC 1081
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1101 AAAGAGAGGACTATTTGGAGCTATAGCAGGTTTTATAGAGGGAGGATGGC 1150
              .         .         .         .         .
1082 AGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGG 1131
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1151 AGGGAATGGTAGATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGG 1200
              .         .         .         .         .
1132 AGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCAATAGATGGAGT 1181
     ||||||||||||||||||||||||||||||||||||||||| ||||||||
1201 AGTGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCAATGGATGGAGT 1250
              .         .         .         .         .
1182 CACCAATAAGGTCAACTCAATCATTGACAAAATGAACACTCAGTTTGAGG 1231
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1251 CACCAATAAGGTCAACTCAATCATTGACAAAATGAACACTCAGTTTGAGG 1300
              .         .         .         .         .
1232 CCGTTGGAAGGGAATTTAATAACTTAGAAAGGAGAATAGAGAATTTAAAC 1281
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1301 CCGTTGGAAGGGAATTTAATAACTTAGAAAGGAGAATAGAGAATTTAAAC 1350
              .         .         .         .         .
1282 AAGAAGATGGAAGACGGGTTTCTAGATGTCTGGACTTATAATGCCGAACT 1331
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1351 AAGAAGATGGAAGACGGGTTTCTAGATGTCTGGACTTATAATGCCGAACT 1400
              .         .         .         .         .
1332 TCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAATG 1381
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1401 TCTGGTTCTCATGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAATG 1450
              .         .         .         .         .
1382 TTAAGAACCTCTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAG 1431
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1451 TTAAGAACCTCTACGACAAGGTCCGACTACAGCTTAGGGATAATGCAAAG 1500
              .         .         .         .         .
1432 GAGCTGGGTAACGGTTGTTTCGAGTTCTATCACAAATGTGATAATGAATG 1481
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1501 GAGCTGGGTAACGGTTGTTTCGAGTTCTATCACAAATGTGATAATGAATG 1550
              .         .         .         .         .
1482 TATGGAAAGTATAAGAAACGGAACGTACAACTATCCGCAGTATTCAGAAG 1531
     |||||||||||||||||||||||||| |||||||||||||||||||||||
1551 TATGGAAAGTATAAGAAACGGAACGTGCAACTATCCGCAGTATTCAGAAG 1600
              .         .         .         .         .
1532 AAGCAAGATTAAAAAGAGAGGAAATAAGTGGGGTAAAATTGGAATCAATA 1581
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1601 AAGCAAGATTAAAAAGAGAGGAAATAAGTGGGGTAAAATTGGAATCAATA 1650
              .         .         .         .         .
1582 GGAACTTACCAAATACTGTCAATTTATTCAACAGTGGCGAGTTCCCTAGC 1631
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1651 GGAACTTACCAAATACTGTCAATTTATTCAACAGTGGCGAGTTCCCTAGC 1700
              .         .         .         .         .
1632 ACTGGCAATCATGATGGCTGGTCTATCTTTATGGATGTGCTCCAATGGAT 1681
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1701 ACTGGCAATCATGATGGCTGGTCTATCTTTATGGATGTGCTCCAATGGAT 1750
```

```
                .         .         .         .         .
1682 CGTTACAATGCAGAATTTGCATtTAA........................ 1707
     ||||||||||||||||||||||||||
1751 CGTTACAATGCAGAATTTGCATTTAAAAGCTTTAAGGGCGAATTCCAGCA 1800
                          .
```

## Amino Acid Sequence Alignment of Hemagglutinin

```
pHA5   1 MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILE 50 seq id 57
         ||||||||||||||||||||||||||||||||||||||||||||||||||
wt     1 MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILE 50 seq id 58
                  .         .         .         .         .
      51 KTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKAN 100
         ||||||||||||||||||||||||||||||||||||||||||||||||||
      51 KTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKAN 100
                  .         .         .         .         .
     101 PTNDLCYPGSFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSA 150
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     101 PTNDLCYPGSFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEASSGVSSA 150
                  .         .         .         .         .
     151 CPYLGSPSFFRNVVWLIKKNSTYPTIKKSYNNTNQEDLLVLWGIHHPNDA 200
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     151 CPYLGSPSFFRNVVWLIKKNSTYPTIKKSYNNTNQEDLLVLWGIHHPNDA 200
                  .         .         .         .         .
     201 AEQTRLYQNPTTYISIGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILK 250
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     201 AEQTRLYQNPTTYISIGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILK 250
                  .         .         .         .         .
     251 PNDAINFESNGNFIAPEYAYKIVKKGDSAIMKSELEYGNCNTKCQTPMGA 300
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     251 PNDAINFESNGNFIAPEYAYKIVKKGDSAIMKSELEYGNCNTKCQTPMGA 300
                  .         .         .         .         .
     301 INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRESRRKKRGLFG 350
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     301 INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRESRRKKRGLFG 350
                  .         .         .         .         .
     351 AIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAMDGVTNKVNS 400
         |||||||||||||||||||||||||||||||||||||||||.|||||||||
     351 AIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNS 400
                  .         .         .         .         .
     401 IIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN 450
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     401 IIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN 450
                  .         .         .         .         .
     451 ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESIRN 500
         ||||||||||||||||||||||||||||||||||||||||||||||||||
     451 ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESIRN 500
                  .         .         .         .         .
     501 GTCNYPQYSEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMMA 550
         || |||||||||||||||||||||||||||||||||||||||||||||||
     501 GTYNYPQYSEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMMA 550
                  .
     551 GLSLWMCSNGSLQCRICI. 568
         ||||||||||||||||||
     551 GLSLWMCSNGSLQCRICI* 569
```

## Example 26

**Generation of influenza A/Indonesia/5/05 HA, NA, and M1 genes optimized for efficient expression in Sf9 cells.**

**[00255]** The following polypeptides were derived from codon-optimized nucleotides corresponding to the Indonesia/5/05 HA gene (see example 31). The codon-optimized nucleotides were designed and produced (Geneart GMBH, Regensburg, FRG) according to the methods disclosed in US patent publication 2005/0118191, herein incorporated by reference in its entirety for all proposes. See Example 31 for nucleic acid sequences

```
Vac2-hac-opt (unmodified aa sequence) (SEQ ID 27)
MEKIVLLLAI VSLVKSDQIC IGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PTNDLCYPGS FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYLGSPSFF RNVVWLIKKN STYPTIKKSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISIGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI MKSELEYGNC NTKCQTPMGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE SRRKKRGLFG
AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESIRN
GTYNYPQYSE EARLKREEIS GVKLESIGTY QILSIYSTVA SSLALAIMMA
GLSLWMCSNG SLQCRICI*

Vac2-hac-spc-opt
(modified, signal peptide from Chitinase, underlined) (SEQ ID 28)
Mplykllnvlwlvavsnaip DQICIGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PTNDLCYPGS FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYLGSPSFF RNVVWLIKKN STYPTIKKSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISIGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI MKSELEYGNC NTKCQTPMGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE SRRKKRGLFG
AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESIRN
GTYNYPQYSE EARLKREEIS GVKLESIGTY QILSIYSTVA SSLALAIMMA
GLSLWMCSNG SLQCRICI*

Vac2-hac-sph9-opt (modified, signal peptide from H9, underlined)
(SEQ ID 29)
METISLITIL LVVTASNA DQICIGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PTNDLCYPGS FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYLGSPSFF RNVVWLIKKN STYPTIKKSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISIGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI MKSELEYGNC NTKCQTPMGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE SRRKKRGLFG
AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
```

```
ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESIRN
GTYNYPQYSE EARLKREEIS GVKLESIGTY QILSIYSTVA SSLALAIMMA
GLSLWMCSNG SLQCRICI*
```

```
Vac2-hac-cs-opt (- is the modified cleavage site) (SEQ ID 30)
MEKIVLLLAI VSLVKSDQIC IGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PTNDLCYPGS FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYLGSPSFF RNVVWLIKKN STYPTIKKSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISIGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI MKSELEYGNC NTKCQTPMGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE S----RGLFG
AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESIRN
GTYNYPQYSE EARLKREEIS GVKLESIGTY QILSIYSTVA SSLALAIMMA
GLSLWMCSNG SLQCRICI*
```

**[00256]** The following polypeptides corresponding to unmodified, codon-optimized NA and M1 genes where also synthesized.

```
Vac2-naj-opt (neuraminidase) (SEQ ID 31)
MNPNQKIITI GSICMVIGIV SLMLQIGNMI SIWVSHSIQT GNQHQAESIS
NTNPLTEKAV ASVTLAGNSS LCPIRGWAVH SKDNNIRIGS KGDVFVIREP
FISCSHLECR TFFLTQGALL NDKHSNGTVK DRSPHRTLMS CPVGEAPSPY
NSRFESVAWS ASACHDGTSW LTIGISGPDN EAVAVLKYNG IITDTIKSWR
NNILRTQESE CACVNGSCFT VMTDGPSDGQ ASYKIFKMEK GKVVKSVELD
APNYHYEECS CYPDAGEITC VCRDNWHGSN RPWVSFNQNL EYQIGYICSG
VFGDNPRPND GTGSCGPMSP NGAYGVKGFS FKYGNGVWIG RTKSTNSRSG
FEMIWDPNGW TGTDSSFSVK QDIVAITDWS GYSGSFVQHP ELTGLDCIRP
CFWVELIRGR PKESTIWTSG SSISFCGVNS DTVSWSWPDG AELPFTIDK*
```

```
Vac2-mc-opt (matrix) (SEQ ID 32)
MSLLTEVETY VLSIIPSGPL KAEIAQKLED VFAGKNTDLE ALMEWLKTRP
ILSPLTKGIL GFVFTLTVPS ERGLQRRRFV QNALNGNGDP NNMDRAVKLY
KKLKREITFH GAKEVSLSYS TGALASCMGL IYNRMGTVTT EVAFGLVCAT
CEQIADSQHR SHRQMATITN PLIRHENRMV LASTTAKAME QMAGSSEQAA
EAMEVANQAR QMVQAMRTIG THPNSSAGLR DNLLENLQAY QKRMGVQMQR
FK*
```

**[00257]** The synthetic, codon-optimized HA, NA, and M1 genes were subcloned into pFastBac1 transfer plasmid using *BamHI* and *HindIII* sites, as described above. Recombinant bacmids for expression in Sf9 cells of synthetic HA, NA, M1 genes were generated as described above, using *E.coli* strain DH10Bac (Invitrogen).

**Example 24**

**Cloning of Clade 1 A/Viet Nam/1203/04 (H5N1) influenza virus by RT-PCR**

[00258] The HA, NA and M1 genes were cloned by RT-PCR according to the above describes method. The below sequences are comparisons of the published gene compared to the cloned genes.

The HA gene for Clade 1 A/Viet Nam/1203/04 (H5N1)

```
Upper Lane: Acc #AY818135 HA gene (SEQ ID 36)
Lower Lane: Novavax's A/Vietnam/1203/2004 (H5N1) HA gene  (SEQ ID 37)

      1 .........................................ATGGAGAAAA 10
                                                 ||||||||||
    301 AGTGTGATGGATATCTGCAGAATTCGCCCTTAGGCGCGCCATGGAGAAAA 350
                  .         .         .         .         .
     11 TAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC 60
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    351 TAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTAAAAGTGATCAGATTTGC 400
                  .         .         .         .         .
     61 ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGA 110
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    401 ATTGGTTACCATGCAAACAACTCGACAGAGCAGGTTGACACAATAATGGA 450
                  .         .         .         .         .
    111 AAAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGAAACACA 160
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    451 AAAGAACGTTACTGTTACACATGCCCAAGACATACTGGAAAAGAAACACA 500
                  .         .         .         .         .
    161 ACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGAT 210
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    501 ACGGGAAGCTCTGCGATCTAGATGGAGTGAAGCCTCTAATTTTGAGAGAT 550
                  .         .         .         .         .
    211 TGTAGCGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCAT 260
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    551 TGTAGCGTAGCTGGATGGCTCCTCGGAAACCCAATGTGTGACGAATTCAT 600
                  .         .         .         .         .
    261 CAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAATCCAGTCAATG 310
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    601 CAATGTGCCGGAATGGTCTTACATAGTGGAGAAGGCCAATCCAGTCAATG 650
                  .         .         .         .         .
    311 ACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA 360
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    651 ACCTCTGTTACCCAGGGGATTTCAATGACTATGAAGAATTGAAACACCTA 700
                  .         .         .         .         .
    361 TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTC 410
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    701 TTGAGCAGAATAAACCATTTTGAGAAAATTCAGATCATCCCCAAAAGTTC 750
                  .         .         .         .         .
    411 TTGGTCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACC 460
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    751 TTGGTCCAGTCATGAAGCCTCATTAGGGGTGAGCTCAGCATGTCCATACC 800
                  .         .         .         .         .
    461 AGGGAAAGTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAGAAC 510
        ||||||||||||||||||||||||||||||||||||||||||||||||||
    801 AGGGAAAGTCCTCCTTTTTCAGAAATGTGGTATGGCTTATCAAAAAGAAC 850
                  .         .         .         .         .
    511 AGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAACCAAGAAGA 560
        ||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
 851 AGTACATACCCAACAATAAAGAGGAGCTACAATAATACCAACCAAGAAGA 900
                    .         .         .         .         .
 561 TCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCGGCAGAGCAGA 610
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 901 TCTTTTGGTACTGTGGGGGATTCACCATCCTAATGATGCGGCAGAGCAGA 950
                    .         .         .         .         .
 611 CAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA 660
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 951 CAAAGCTCTATCAAAACCCAACCACCTATATTTCCGTTGGGACATCAACA 1000
                    .         .         .         .         .
 661 CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGG 710
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1001 CTAAACCAGAGATTGGTACCAAGAATAGCTACTAGATCCAAAGTAAACGG 1050
                    .         .         .         .         .
 711 GCAAAGTGGAAGGATGGAGTTCTTCTGGACAATTTTAAAGCCGAATGATG 760
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1051 GCAAAGTGGAAGGATGGAGTTCTTCTGGACAATTTTAAAGCCGAATGATG 1100
                    .         .         .         .         .
 761 CAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATAC 810
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1101 CAATCAACTTCGAGAGTAATGGAAATTTCATTGCTCCAGAATATGCATAC 1150
                    .         .         .         .         .
 811 AAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTGGAATA 860
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1151 AAAATTGTCAAGAAAGGGGACTCAACAATTATGAAAAGTGAATTGGAATA 1200
                    .         .         .         .         .
 861 TGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTA 910
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1201 TGGTAACTGCAACACCAAGTGTCAAACTCCAATGGGGGCGATAAACTCTA 1250
                    .         .         .         .         .
 911 GCATGCCATTCCACAATATACACCCTCTCACCATTGGGGAATGCCCCAAA 960
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1251 GCATGCCATTCCACAATATACACCCTCTCACCATTGGGGAATGCCCCAAA 1300
                    .         .         .         .         .
 961 TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCC 1010
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1301 TATGTGAAATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCC 1350
                    .         .         .         .         .
1011 TCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAG 1060
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1351 TCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTATTTGGAGCTATAGCAG 1400
                    .         .         .         .         .
1061 GTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGGTAC 1110
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1401 GTTTTATAGAGGGAGGATGGCAGGGAATGGTAGATGGTTGGTATGGGTAC 1450
                    .         .         .         .         .
1111 CACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCAC 1160
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1451 CACCATAGCAATGAGCAGGGGAGTGGGTACGCTGCAGACAAAGAATCCAC 1500
                    .         .         .         .         .
1161 TCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACA 1210
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1501 TCAAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTCGATCATTGACA 1550
                    .         .         .         .         .
1211 AAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1260
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1551 AAATGAACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAGAA 1600
                    .         .         .         .         .
1261 AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGT 1310
     ||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
1601 AGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATGT 1650
              .         .         .         .         .
1311 CTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTC 1360
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1651 CTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAAAATGAGAGAACTC 1700
              .         .         .         .         .
1361 TAGACTTTCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTA 1410
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1701 TAGACTTTCATGACTCAAATGTCAAGAACCTTTACGACAAGGTCCGACTA 1750
              .         .         .         .         .
1411 CAGCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTA 1460
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1751 CAGCTTAGGGATAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTA 1800
              .         .         .         .         .
1461 TCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAATGGAACGTATG 1510
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1801 TCATAAATGTGATAATGAATGTATGGAAAGTGTAAGAAATGGAACGTATG 1850
              .         .         .         .         .
1511 ACTACCCGCAGTATTCAGAAGAAGCGAGACTAAAAAGAGAGGAAATAAGT 1560
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1851 ACTACCCGCAGTATTCAGAAGAAGCGAGACTAAAAAGAGAGGAAATAAGT 1900
              .         .         .         .         .
1561 GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTC 1610
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1901 GGAGTAAAATTGGAATCAATAGGAATTTACCAAATACTGTCAATTTATTC 1950
              .         .         .         .         .
1611 TACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCT 1660
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1951 TACAGTGGCGAGTTCCCTAGCACTGGCAATCATGGTAGCTGGTCTATCCT 2000
              .         .         .         .         .
1661 TATGGATGTGCTCCAATGGATCGTTACAATGCAGAATTTGCATTTAA... 1707
     ||||||||||||||||||| |||||||||||||||||||||||||||
2001 TATGGATGTGCTCCAATGGGTCGTTACAATGCAGAATTTGCATTTAAGCG 2050
```

Comparison of the NA genes.

The NA gene for Clade 1 A/Viet Nam/1203/04 (H5N1) (SEQ ID 39)

H5N1naLANL ISDN 38704 x NA_Viet1203_Lark(NVAX) (SEQ ID 38)

```
              .         .         .         .         .
   1 .....ATGAATCCAAATCAGAAGATAATAACCATCGGATCAATCTGTATG 45
          |||||||||||||||||||||||||||||||||||||||||||||
 451 CCGGGATGAATCCAAATCAGAAGATAATAACCATCGGATCAATCTGTATG 500
              .         .         .         .         .
  46 GTAACTGGAATAGTTAGCTTAATGTTACAAATTGGGAACATGATCTCAAT 95
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 501 GTAACTGGAATAGTTAGCTTAATGTTACAAATTGGGAACATGATCTCAAT 550
              .         .         .         .         .
  96 ATGGGTCAGTCATTCAATTCACACAGGGAATCAACACCAATCTGAACCAA 145
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 551 ATGGGTCAGTCATTCAATTCACACAGGGAATCAACACCAATCTGAACCAA 600
              .         .         .         .         .
 146 TCAGCAATACTAATTTTCTTACTGAGAAAGCTGTGGCTTCAGTAAAATTA 195
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 601 TCAGCAATACTAATTTTCTTACTGAGAAAGCTGTGGCTTCAGTAAAATTA 650
              .         .         .         .         .
 196 GCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGCTGTATACAGTAA 245
```

```
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 651 GCGGGCAATTCATCTCTTTGCCCCATTAACGGATGGGCTGTATACAGTAA 700
               .         .         .         .         .
 246 GGACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAG 295
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 701 GGACAACAGTATAAGGATCGGTTCCAAGGGGGATGTGTTTGTTATAAGAG 750
               .         .         .         .         .
 296 AGCCGTTCATCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTTGACT 345
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 751 AGCCGTTCATCTCATGCTCCCACTTGGAATGCAGAACTTTCTTTTTGACT 800
               .         .         .         .         .
 346 CAGGGAGCCTTGCTGAATGACAAGCACTCCAATGGGACTGTCAAAGACAG 395
     |||||||||| |||||||||||||||||||||||||||||||||||||||
 801 CAGGGAGCCTCGCTGAATGACAAGCACTCCAATGGGACTGTCAAAGACAG 850
               .         .         .         .         .
 396 AAGCCCTCACAGAACATTAATGAGTTGTCCTGTGGGTGAGGCTCCCTCCC 445
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 851 AAGCCCTCACAGAACATTAATGAGTTGTCCTGTGGGTGAGGCTCCCTCCC 900
               .         .         .         .         .
 446 CATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAGTGCTTGCCAT 495
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 901 CATATAACTCAAGGTTTGAGTCTGTTGCTTGGTCAGCAAGTGCTTGCCAT 950
               .         .         .         .         .
 496 GATGGCACCAGTTGGTTGACGATTGGAATTTCTGGCCCAGACAATGGGGC 545
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 951 GATGGCACCAGTTGGTTGACGATTGGAATTTCTGGCCCAGACAATGGGGC 1000
               .         .         .         .         .
 546 TGTGGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTT 595
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1001 TGTGGCTGTATTGAAATACAATGGCATAATAACAGACACTATCAAGAGTT 1050
               .         .         .         .         .
 596 GGAGGAACAACATACTGAGAACTCAAGAGTCTGAATGTGCATGTGTAAAT 645
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1051 GGAGGAACAACATACTGAGAACTCAAGAGTCTGAATGTGCATGTGTAAAT 1100
               .         .         .         .         .
 646 GGCTCTTGCTTTACTGTAATGACTGACGGACCAAGTAATGGTCAGGCATC 695
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1101 GGCTCTTGCTTTACTGTAATGACTGACGGACCAAGTAATGGTCAGGCATC 1150
               .         .         .         .         .
 696 ACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATCAGTCGAAT 745
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1151 ACATAAGATCTTCAAAATGGAAAAAGGGAAAGTGGTTAAATCAGTCGAAT 1200
               .         .         .         .         .
 746 TGGATGCTCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTAATGCC 795
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1201 TGGATGCTCCTAATTATCACTATGAGGAATGCTCCTGTTATCCTAATGCC 1250
               .         .         .         .         .
 796 GGAGAAATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCC 845
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1251 GGAGAAATCACATGTGTGTGCAGGGATAATTGGCATGGCTCAAATCGGCC 1300
               .         .         .         .         .
 846 ATGGGTATCTTTCAATCAAAATTTGGAGTATCAAATAGGATATATATGCA 895
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1301 ATGGGTATCTTTCAATCAAAATTTGGAGTATCAAATAGGATATATATGCA 1350
               .         .         .         .         .
 896 GTGGAGTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGTAGTTGT 945
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1351 GTGGAGTTTTCGGAGACAATCCACGCCCCAATGATGGAACAGGTAGTTGT 1400
               .         .         .         .         .
 946 GGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAA 995
```

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     ||||||||||||||||||||||||||||||||||||||||||||||||||
1401 GGTCCGGTGTCCTCTAACGGGGCATATGGGGTAAAAGGGTTTTCATTTAA 1450
              .         .         .         .         .
 996 ATACGGCAATGGTGTCTGGATCGGGAGAACCAAAAGCACTAATTCCAGGA 1045
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1451 ATACGGCAATGGTGTCTGGATCGGGAGAACCAAAAGCACTAATTCCAGGA 1500
              .         .         .         .         .
1046 GCGGCTTTGAAATGATTTGGGATCCAAATGGGTGGACTGAAACGGACAGT 1095
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1501 GCGGCTTTGAAATGATTTGGGATCCAAATGGGTGGACTGAAACGGACAGT 1550
              .         .         .         .         .
1096 AGCTTTTCAGTGAAACAAGATATCGTAGCAATAACTGATTGGTCAGGATA 1145
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1551 AGCTTTTCAGTGAAACAAGATATCGTAGCAATAACTGATTGGTCAGGATA 1600
              .         .         .         .         .
1146 TAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGACTAGATTGCATAA 1195
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1601 TAGCGGGAGTTTTGTCCAGCATCCAGAACTGACAGGACTAGATTGCATAA 1650
              .         .         .         .         .
1196 GACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAGCACA 1245
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1651 GACCTTGTTTCTGGGTTGAGTTGATCAGAGGGCGGCCCAAAGAGAGCACA 1700
              .         .         .         .         .
1246 ATTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGTGTAAATAGTGACAC 1295
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1701 ATTTGGACTAGTGGGAGCAGCATATCTTTTTGTGGTGTAAATAGTGACAC 1750
              .         .         .         .         .
1296 TGTGGGTTGGTCTTGGCCAGACGGTGCCGAGTTGCCATTCACCATTGACA 1345
     |||||||||||||||||||||||||| |||||||||||||||||||||||
1751 TGTGGGTTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCACCATTGACA 1800
              .         .         .         .         .
1346 AGTAG............................................. 1350
     |||||
1801 AGTAGGGGCCCTCGAGTAAGGGCGAATTCCAGCACACTGGCGGCCGTTAC 1850
                        .
```

Comparisons of the M1 genes.

```
The M1 gene for Clade 1 A/Viet Nam/1203/04 (H5N1) (SEQ ID 40)
H5N1m1Lanl ISDN39958 x M1_Viet1203_Lark(NVAX) (SEQ ID 41)
              .         .         .         .         .
   1 ..................................ATGAGTCTTCTAACCG 16
                                   ||||||||||||||||
 301 ATATCTGCAGAATTCGCCCTTAGAATTCGACGTCATGAGTCTTCTAACCG 350
              .         .         .         .         .
  17 AGGTCGAAACGTACGTTCTCTCTATCATCCCGTCAGGCCCCCTCAAAGCC 66
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 351 AGGTCGAAACGTACGTTCTCTCTATCATCCCGTCAGGCCCCCTCAAAGCC 400
              .         .         .         .         .
  67 GAGATCGCACAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGATCT 116
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 401 GAGATCGCACAGAAACTTGAAGATGTCTTTGCAGGAAAGAACACCGATCT 450
              .         .         .         .         .
 117 CGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACCTCTGA 166
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 451 CGAGGCTCTCATGGAGTGGCTAAAGACAAGACCAATCCTGTCACCTCTGA 500
              .         .         .         .         .
 167 CTAAAGGGATTTTGGGATTTGTATTCACGCTCACCGTGCCCAGTGAGCGA 216
     ||||||||||||||||||||||||||||||||||||||||||||||||||
```

```
 501 CTAAAGGGATTTTGGGATTTGTATTCACGCTCACCGTGCCCAGTGAGCGA 550
              .         .         .         .         .
 217 GGACTGCAGCGTAGACGCTTTGTCCAGAATGCCCTAAATGGAAATGGAGA 266
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 551 GGACTGCAGCGTAGACGCTTTGTCCAGAATGCCCTAAATGGAAATGGAGA 600
              .         .         .         .         .
 267 TCCAAATAATATGGATAGGGCAGTTAAGCTATATAAGAAGCTGAAAAGAG 316
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 601 TCCAAATAATATGGATAGGGCAGTTAAGCTATATAAGAAGCTGAAAAGAG 650
              .         .         .         .         .
 317 AAATAACATTCCATGGGGCTAAGGAGGTCGCACTCAGCTACTCAACCGGT 366
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 651 AAATAACATTCCATGGGGCTAAGGAGGTCGCACTCAGCTACTCAACCGGT 700
              .         .         .         .         .
 367 GCACTTGCCAGTTGCATGGGTCTCATATACAACAGGATGGGAACGGTGAC 416
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 701 GCACTTGCCAGTTGCATGGGTCTCATATACAACAGGATGGGAACGGTGAC 750
              .         .         .         .         .
 417 TACGGAAGTGGCTTTTGGCCTAGTGTGTGCCACTTGTGAGCAGATTGCAG 466
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 751 TACGGAAGTGGCTTTTGGCCTAGTGTGTGCCACTTGTGAGCAGATTGCAG 800
              .         .         .         .         .
 467 ATTCACAGCATCGGTCTCACAGACAGATGGCAACTATCACCAACCCACTA 516
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 801 ATTCACAGCATCGGTCTCACAGACAGATGGCAACTATCACCAACCCACTA 850
              .         .         .         .         .
 517 ATCAGACATGAGAACAGAATGGTGCTGGCCAGCACTACAGCTAAGGCTAT 566
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 851 ATCAGACATGAGAACAGAATGGTGCTGGCCAGCACTACAGCTAAGGCTAT 900
              .         .         .         .         .
 567 GGAGCAGATGGCGGGATCAAGTGAGCAGGCAGCGGAAGCCATGGAGATCG 616
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 901 GGAGCAGATGGCGGGATCAAGTGAGCAGGCAGCGGAAGCCATGGAGATCG 950
              .         .         .         .         .
 617 CTAATCAGGCTAGGCAGATGGTGCAGGCAATGAGGACAATTGGGACTCAT 666
     ||||||||||||||||||||||||||||||||||||||||||||||||||
 951 CTAATCAGGCTAGGCAGATGGTGCAGGCAATGAGGACAATTGGGACTCAT 1000
              .         .         .         .         .
 667 CCTAACTCTAGTGCTGGTCTGAGAGATAATCTTCTTGAAAATTTGCAGGC 716
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1001 CCTAACTCTAGTGCTGGTCTGAGAGATAATCTTCTTGAAAATTTGCAGGC 1050
              .         .         .         .
 717 CTACCAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTGA
     |||||||||||||||||||||||||||||||||||||||||||
1051 CTACCAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTGA
```

[00259] All the sequences were cloned and analyzed according to the dislcosed methods above.

**Example 25**

**Generation of Clade 1 H5N1 influenza A/Viet Nam/1203/04 HA, NA, and M1 genes optimized for efficient expression in Sf9 cells**

[00260] The following polypeptides were derived from codon-optimized nucleotides corresponding to A/Viet Nam/1203/04. The nucleotides were designed and synthesized (Geneart GMBH, Regensburg, FRG) as disclosed above (see Example 24).

VN1203-ha-cs-opt (modified cleavage site, underlined) (SEQ ID 33)

```
MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTH
AQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPA
NDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKNSWSSHEASLGVSSACPYQGKSSFF
RNVVWLIKKNNAYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGT
STLNQRLVPKIATRSKVNGQNGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKG
DSAIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLR
NSPQRET----RGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAID
GVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN
ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQY
SEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMVAGLSLWMCSNGSLQCRI
CI*
```

VN1203-ha-spc-opt (modified signal peptide, underlined) (SEQ ID 34)

```
Mplykllnvlwlvavsnaip DQICIGYHANNSTEQVDTIMEKNVTVTH
AQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPA
NDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKNSWSSHEASLGVSSACPYQGKSSFF
RNVVWLIKKNNAYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGT
STLNQRLVPKIATRSKVNGQNGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKG
DSAIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLR
NSPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAID
GVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN
ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQY
SEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMVAGLSLWMCSNGSLQCRI
CI*
```

VN1203-ha-sph9-opt (The signal peptide and cleavage site are shaded) (SEQ ID 35)

```
METISLITIL LVVTASNA DQICIGYHANNSTEQVDTIMEKNVTVTH
AQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPA
NDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKNSWSSHEASLGVSSACPYQGKSSFF
RNVVWLIKKNNAYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYISVGT
STLNQRLVPKIATRSKVNGQNGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKG
DSAIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLR
NSPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAID
GVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMEN
ERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQY
SEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMVAGLSLWMCSNGSLQCRI
CI*
```

## Example 26

## H5N1 Vietnam/1203/2003 VLP Immunogenicity (Extreme Dose Sparing)

[00261] BALB/C mice were immunized intramuscularly and intranasally with H5N1 VLPs at very low doses of VLPs (0.2, 0.04, 0.008, 0.0016 µg HA /dose), Mice were bled on days 0, 21 and 35. The mice were given a boost on day 21. The serum was assayed for anti-HA

antibodies by the hemagglutination inhibition assay (HI) using turkey RBCs and influenza virus using an ELISA. Results of this study are shown in Figures 24 and 25.

**[00262]** The results indicate that a robust overall immune response was observed when the VLPs were administered intramuscularly at very lose doses. The robustness of the response was similar to control at 3.0 and 0.6 μg HA /dose. These data show see a true dose response and the antibody response to 0.2 μg of VLP is greater than 3.0 μg of rHA protein. Although the response was not as robust for the intranasal administration, a dose of VLPs at 0.2 μg HA /dose did induce a robust response. The ELISA titer with the 0.2 μg dose in this experiment is similar to the 0.12 μg dose of the H3N2 VLP vaccine in previous experiments, see above.

**Example 27**

**Challenge studies**

After inoculating BALB/c mice with VLPs at concentrations of 3 μg, 0.6 μg 0.12 μg and 0.02 μg of H3N2 VLPs intramuscularly and intranasally (total HA dose), mice were challenged with influenza virus A/Aichi/268x31. The results of this study are shown on Figures 27 and 28. These data show that there is a decrease in weight in all vaccinated animals, however the animals that were vaccinated with 3.0 μg and 0.12 μg of VLPs recovered quicker than the other animals in both intramuscular and intranasal vaccinations. The intranasal doses provided enhanced protection.

**Example 29**

**Challenge studies (ferrets)**

In this study, ferrets were vaccinated with H9N2 VLPs. There were a total of 18 ferrets in the challenge study: 6 mock vaccinated, 6 vaccinated with medium dose (1.5 μg), and 6 vaccinated with high dose (15.0 μg) intramuscularly. Next, ferrets were challenged with $10^6$ $EID_{50}$ of A/HK/1073/99 intranasally. Nasal washes were collected on days 1, 3, 5 and 7. The virus in the nasal washes was titered on days 3, 5 and 7 for all animals. These data are represented on Table 2 and in Figure 29. These data show that by day 7, all of the vaccinated animals had no detectable virus in nasal washes while the mock group had detectable viral titers.

TABLE 2. Wild Type Virus Titers (log 10/ml) in Ferrets after viral challenge

Group: Placebo Mock Control (n=6)

| Ferret | Day 3 | Day 5 | Day 7 |
|---|---|---|---|
| 4512 | 7 | 5.5 | 3.5 |
| 4524 | 6.5 | 6.75 | 1.98 |
| 4525 | 7.5 | 6.5 | 6.75 |
| 4526 | 7.5 | 7.25 | 3.5 |
| 4527 | 6.75 | 7.25 | 2.5 |
| 4528 | 7.5 | 6.25 | 2.75 |
| Mean | 7.125 | 6.583333 | 3.496667 |
| Std. Dev. | 0.44017 | 0.66458 | 1.699137 |
| | | | |

Group: Low Dose

| Ferret | Day 3 | Day 5 | Day 7 |
|---|---|---|---|
| 3916 | 6.75 | 2.75 | 1.5 |
| 3917 | 7.5 | 5.5 | 1.5 |
| 3918 | 7.5 | 6.5 | 1.5 |
| 3919 | 5.5 | 3 | 1.5 |
| 3920 | 6.75 | 2.25 | 1.5 |
| 3921 | 6.5 | 3.5 | 1.5 |
| Avg | 6.75 | 3.916667 | 1.5 |
| Std Dev | 0.74162 | 1.693123 | 0 |
| | | | |

Group: High Dose

| Ferret | Day 3 | Day 5 | Day 7 |
|---|---|---|---|
| 3922 | 6.5 | 2.75 | 1.5 |
| 3923 | 6.25 | 3.75 | 1.5 |
| 3924 | 5.75 | 1.5 | 1.5 |
| 3925 | 6.5 | 4.75 | 1.5 |
| 3926 | 6.25 | 3.5 | 1.5 |
| 3927 | 5.75 | 1.5 | 1.5 |
| Avg. | 6.166667 | 2.958333 | 1.5 |
| Std Dev | 0.341565 | 1.298236 | 0 |

**Example 30**

**Mice Intramuscular and Intranasal Inoculation Studies**

**[00263]** Mice were inoculated with A/Fujian/411/2002 (H3N2) VLPs at concentrations of 3 μg, 0.6 μg 0.12 μg or 0.024 μg (total HA dose) intramuscularly or intranasally at day 0 and were boosted 3 weeks later. Control mice were inoculated with formalin inactivated A/Wyoming (Fujian-Like, vaccine strain) or PBS. Sera were collected from the inoculated mice at weeks 0, 3, 5 and 8. The collected sera were assayed for anti-HA antibodies by the hemagglutination inhibition assay (HI) for anti-influenza antibodies by ELISA. The assay was conducted using A/Fujian/411/2002, A/Panama/2007/99, A/Wyoming/3/03 and A/New

York/55/2004 influenza virus strains of H3N2. Results of this study are shown on Figures 30 A-H. These data indicate the H3N2 VLPs induced antibodies against the parent A/Fujian/411/2002 strains of influenza virus and against other H3N2 strains. These data also indicate that the titers in intranasally inoculated mice rise later than intramuscularly inoculated mice. However, the intranasal titers are higher than intramuscular titers after about 8 weeks. In addition, titers to the inactivated virus antigen appear to be comparable to the VLP at equivalent doses following intramuscular inoculation. However, the inactivated antigen does not appear to be as immunogenic following intranasal inoculation, nor is it as broadly protective following intranasal inoculation.

**Example 31**

**Generation of Clade 2 H5N1 influenza HA, NA, and M1 genes optimized for efficient expression in Sf9 cells**

The following optimized nucleotides and polypeptides corresponding to HA, NA and M1 of Clade 2 H5N1 viruses, A A/Indonesia/5/05, A/Bar headed goose/Qinghai/1A/2005 and A/Anhui/1/2005, were designed and synthesized (Geneart GMBH, Regensburg, FRG) as disclosed above. The optimized nucleotides and polypeptides are listed below. In order to make VLPs, A/Anhui HA can be expressed with A/Indonesia NA and M1. For VLPs comprising A/Quinghai HA and NA, A/Indonesia M1 gene can be co-expressed with A/Quinghai HA and NA.

```
A/INDONESIA/5/05

A/INDONESIA Optimized HA (Start and stop codon are underlined)  (SEQ ID 42)

GGTACCGGATCCGCCACCATGGAGAAGATCGTGCTGCTGCTGGCTATCGTGTCCCTGGTG
AAGTCCGACCAGATCTGCATCGGTTACCACGCTAACAACTCCACCGAGCAGGTGGACACC
ATCATGGAGAAGAACGTCACCGTGACCCACGCTCAGGACATCCTCGAAAAGACCCACAAC
GGCAAGCTGTGCGACCTGGACGGTGTCAAGCCCCTGATCCTGCGTGACTGCTCCGTGGCT
GGTTGGCTGCTGGGTAACCCCATGTGCGACGAGTTCATCAACGTGCCCGAGTGGTCCTAC
ATCGTGGAGAAGGCTAACCCCACCAACGACCTGTGCTACCCCGGTTCCTTCAACGACTAC
GAGGAGCTGAAGCACCTGCTGTCCCGTATCAACCACTTCGAGAAGATCCAGATCATCCCC
AAGTCCTCTTGGTCCGACCACGAGGCTTCCTCCGGTGTCTCCTCCGCTTGCCCCTACCTG
GGTTCCCCCTCCTTCTTCCGTAACGTGGTGTGGCTGATCAAGAAGAACTCCACCTACCCC
ACCATCAAGAAGTCCTACAACAACACCAACCAGGAGGACCTGCTGGTCCTGTGGGGTATC
CACCACCCCAACGACGCTGCCGAGCAGACCCGTCTGTACCAGAACCCCACCACCTACATC
TCCATCGGCACCTCCACCCTGAACCAGCGTCTGGTGCCCAAGATCGCTACCCGTTCCAAG
GTGAACGGCCAGTCCGGTCGTATGGAGTTCTTCTGGACCATCCTGAAGCCTAACGACGCT
ATCAACTTCGAGTCCAACGGCAACTTCATCGCTCCCGAGTACGCTTACAAGATCGTGAAG
AAGGGCGACTCCGCTATCATGAAGTCCGAGCTGGAGTACGGTAACTGCAACACCAAGTGC
CAGACCCCCATGGGTGCTATCAACTCCTCCATGCCCTTCCACAACATCCACCCCCTGACC
ATCGGCGAGTGCCCCAAGTACGTGAAGTCCAACCGTCTGGTGCTGGCTACCGGTCTGCGT
```

AACTCCCCCCAGCGCGAGTCCCGTCGTAAGAAGCGTGGTCTGTTCGGCGCTATCGCTGGT
TTCATCGAGGGCGGTTGGCAGGGCATGGTGGACGGATGGTACGGTTACCACCACTCTAAC
GAGCAGGGTTCCGGTTACGCTGCTGACAAGGAGTCCACCCAGAAGGCTATCGACGGCGTC
ACCAACAAGGTGAACTCCATCATCGACAAGATGAACACCCAGTTCGAGGCTGTGGGTCGT
GAGTTCAACAACCTCGAGCGTCGTATCGAGAACCTGAACAAGAAGATGGAGGACGGTTTC
CTGGACGTGTGGACCTACAACGCCGAGCTGCTGGTGCTGATGGAGAACGAGCGTACCCTG
GACTTCCACGACTCCAACGTGAAGAACCTGTACGACAAGGTCCGCCTGCAGCTGCGTGAC
AACGCTAAGGAGCTGGGTAACGGTTGCTTCGAGTTCTACCACAAGTGCGACAACGAGTGC
ATGGAGTCCATCCGTAACGGCACCTACAACTACCCCCAGTACTCCGAGGAGGCTCGTCTG
AAGCGTGAGGAGATCTCCGGCGTGAAGCTCGAGTCCATCGGAACCTACCAGATCCTGTCC
ATCTACTCCACCGTGGCTTCCTCCCTGGCTCTGGCTATCATGATGGCTGGTCTGTCCCTG
TGGATGTGCTCCAACGGTTCCCTGCAGTGCCGTATCTGCATCTAATGAAAGCTTGAGCTC

A/INDONESIA **HA Protein Sequence** (SEQ ID 43)

MEKIVLLLAI VSLVKSDQIC IGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PTNDLCYPGS FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYLGSPSFF RNVVWLIKKN STYPTIKKSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISIGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI MKSELEYGNC NTKCQTPMGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE SRRKKRGLFG
AIAGFIEGGW QGMVDGWYGY HHSNEQGSGY AADKESTQKA IDGVTNKVNS
IIDKMNTQFE AVGREFNNLE RRIENLNKKM EDGFLDVWTY NAELLVLMEN
ERTLDFHDSN VKNLYDKVRL QLRDNAKELG NGCFEFYHKC DNECMESIRN
GTYNYPQYSE EARLKREEIS GVKLESIGTY QILSIYSTVA SSLALAIMMA
GLSLWMCSNG SLQCRICI

A/INDONESIA Optimized HA (cleavage site deleted)
(Start and stop codon are underlined)(SEQ ID 44)

GGATCCGCCACCATGGAGAAGATCGTGCTGCTGCTGGCTATCGTGTCCCTGGTGAAGTCC
GACCAGATCTGCATCGGTTACCACGCTAACAACTCCACCGAGCAGGTGGACACCATCATG
GAGAAGAACGTCACCGTGACCCACGCTCAGGACATCCTCGAAAAGACCCACAACGGCAAG
CTGTGCGACCTGGACGGTGTCAAGCCCCTGATCCTGCGTGACTGCTCCGTGGCTGGTTGG
CTGCTGGGTAACCCCATGTGCGACGAGTTCATCAACGTGCCCGAGTGGTCCTACATCGTG
GAGAAGGCTAACCCCACCAACGACCTGTGCTACCCCGGTTCCTTCAACGACTACGAGGAG
CTGAAGCACCTGCTGTCCCGTATCAACCACTTCGAGAAGATCCAGATCATCCCCAAGTCC
TCTTGGTCCGACCACGAGGCTTCCTCCGGTGTCTCCTCCGCTTGCCCCTACCTGGGTTCC
CCCTCCTTCTTCCGTAACGTGGTGTGGCTGATCAAGAAGAACTCCACCTACCCCACCATC
AAGAAGTCCTACAACAACACCAACCAGGAGGACCTGCTGGTCCTGTGGGGTATCCACCAC
CCCAACGACGCTGCCGAGCAGACCCGTCTGTACCAGAACCCCACCACCTACATCTCCATC
GGCACCTCCACCCTGAACCAGCGTCTGGTGCCCAAGATCGCTACCCGTTCCAAGGTGAAC
GGCCAGTCCGGTCGTATGGAGTTCTTCTGGACCATCCTGAAGCCTAACGACGCTATCAAC
TTCGAGTCCAACGGCAACTTCATCGCTCCCGAGTACGCTTACAAGATCGTGAAGAAGGGC
GACTCCGCTATCATGAAGTCCGAGCTGGAGTACGGTAACTGCAACACCAAGTGCCAGACC
CCCATGGGTGCTATCAACTCCTCCATGCCCTTCCACAACATCCACCCCCTGACCATCGGC
GAGTGCCCCAAGTACGTGAAGTCCAACCGTCTGGTGCTGGCTACCGGTCTGCGTAACTCC
CCCCAGCGCGAGTCCCGTGGTCTGTTCGGCGCTATCGCTGGTTTCATCGAGGGCGGTTGG
CAGGGCATGGTGGACGGATGGTACGGTTACCACCACTCTAACGAGCAGGGTTCCGGTTAC
GCTGCTGACAAGGAGTCCACCCAGAAGGCTATCGACGGCGTCACCAACAAGGTGAACTCC
ATCATCGACAAGATGAACACCCAGTTCGAGGCTGTGGGTCGTGAGTTCAACAACCTCGAG
CGTCGTATCGAGAACCTGAACAAGAAGATGGAGGACGGTTTCCTGGACGTGTGGACCTAC
AACGCCGAGCTGCTGGTGCTGATGGAGAACGAGCGTACCCTGGACTTCCACGACTCCAAC
GTGAAGAACCTGTACGACAAGGTCCGCCTGCAGCTGCGTGACAACGCTAAGGAGCTGGGT
AACGGTTGCTTCGAGTTCTACCACAAGTGCGACAACGAGTGCATGGAGTCCATCCGTAAC
GGCACCTACAACTACCCCCAGTACTCCGAGGAGGCTCGTCTGAAGCGTGAGGAGATCTCC
GGCGTGAAGCTCGAGTCCATCGGAACCTACCAGATCCTGTCCATCTACTCCACCGTGGCT
TCCTCCCTGGCTCTGGCTATCATGATGGCTGGTCTGTCCCTGTGGATGTGCTCCAACGGT

TCCCTGCAGTGCCGTATCTGCATCTAATGAAAGCTT

A/INDONESIA **HA Protein sequence** (SEQ ID 45)

MEKIVLLLAI VSLVKSDQIC IGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PTNDLCYPGS FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYLGSPSFF RNVVWLIKKN STYPTIKKSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISIGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI MKSELEYGNC NTKCQTPMGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQRE SRGLFGAIAG
FIEGGWQGMV DGWYGYHHSN EQGSGYAADK ESTQKAIDGV TNKVNSIIDK
MNTQFEAVGR EFNNLERRIE NLNKKMEDGF LDVWTYNAEL LVLMENERTL
DFHDSNVKNL YDKVRLQLRD NAKELGNGCF EFYHKCDNEC MESIRNGTYN
YPQYSEEARL KREEISGVKL ESIGTYQILS IYSTVASSLA LAIMMAGLSL
WMCSNGSLQC RICI

A/INDONESIA Optimized NA (Start and stop codon are underlined)
(SEQ ID 46)

GGTACCGGATCCGCCACCATGAACCCCAACCAGAAGATCATCACCATCGGCTCCATCTGC
ATGGTGATCGGTATCGTGTCCCTGATGCTGCAGATCGGTAACATGATCTCCATCTGGGTG
TCCCACTCCATCCAGACCGGTAACCAGCACCAGGCTGAGTCCATCTCCAACACCAACCCC
CTGACCGAGAAGGCTGTGGCTTCCGTGACCCTGGCTGGTAACTCCTCCCTGTGCCCCATC
CGTGGTTGGGCTGTGCACTCCAAGGACAACAACATCCGCATCGGTTCCAAGGGTGACGTG
TTCGTGATCCGTGAGCCCTTCATCTCCTGCTCCCACCTCGAGTGCCGTACCTTCTTCCTG
ACCCAAGGTGCTCTGCTGAACGACAAGCACTCCAACGGCACCGTGAAGGACCGTTCCCCC
CACCGTACCCTGATGTCCTGCCCCGTGGGCGAGGCTCCCTCCCCCTACAACTCCCGTTTC
GAGTCCGTGGCTTGGTCCGCTTCCGCTTGCCACGACGGCACCTCTTGGCTGACCATCGGT
ATCTCCGGTCCCGACAACGAGGCTGTCGCTGTGCTGAAGTACAACGGCATCATCACCGAC
ACCATCAAGTCCTGGCGTAACAACATCCTGCGTACCCAGGAGTCCGAGTGCGCTTGCGTG
AACGGTTCCTGCTTCACCGTGATGACCGACGGTCCCTCCGACGGCCAGGCTTCCTACAAG
ATCTTCAAGATGGAGAAGGGCAAGGTGGTGAAGTCCGTGGAGCTGGACGCTCCCAACTAC
CACTACGAGGAGTGCTCTTGCTACCCCGACGCTGGCGAGATCACCTGCGTGTGCCGTGAC
AACTGGCACGGTTCCAACCGTCCCTGGGTGTCCTTCAACCAGAACCTCGAGTACCAGATC
GGTTACATCTGCTCCGGCGTGTTCGGTGACAACCCCCGTCCCAACGACGGAACCGGTTCC
TGCGGTCCCATGTCCCCCAACGGTGCTTACGGTGTCAAGGGCTTCTCCTTCAAGTACGGT
AACGGTGTCTGGATCGGTCGTACCAAGTCCACCAACTCCCGCTCCGGTTTCGAGATGATC
TGGGACCCCAACGGTTGGACCGGCACCGACTCTTCCTTCTCCGTGAAGCAGGACATCGTG
GCTATCACCGACTGGTCCGGTTACTCCGGTTCCTTCGTGCAGCACCCCGAGCTGACCGGT
CTGGACTGCATTCGTCCCTGCTTCTGGGTGGAGCTGATCCGTGGTCGTCCCAAGGAGTCC
ACCATCTGGACCTCCGGCTCCTCCATCTCTTTCTGCGGTGTGAACTCCGACACCGTGTCC
TGGTCCTGGCCCGACGGTGCCGAGCTGCCCTTCACCATCGACAAGTAATGAAAGCTTGAG
CTC

A/INDONESIA **NA Protein sequence** (SEQ ID 47)

MNPNQKIITI GSICMVIGIV SLMLQIGNMI SIWVSHSIQT GNQHQAESIS
NTNPLTEKAV ASVTLAGNSS LCPIRGWAVH SKDNNIRIGS KGDVFVIREP
FISCSHLECR TFFLTQGALL NDKHSNGTVK DRSPHRTLMS CPVGEAPSPY
NSRFESVAWS ASACHDGTSW LTIGISGPDN EAVAVLKYNG IITDTIKSWR
NNILRTQESE CACVNGSCFT VMTDGPSDGQ ASYKIFKMEK GKVVKSVELD
APNYHYEECS CYPDAGEITC VCRDNWHGSN RPWVSFNQNL EYQIGYICSG
VFGDNPRPND GTGSCGPMSP NGAYGVKGFS FKYGNGVWIG RTKSTNSRSG
FEMIWDPNGW TGTDSSFSVK QDIVAITDWS GYSGSFVQHP ELTGLDCIRP
CFWVELIRGR PKESTIWTSG SSISFCGVNS DTVSWSWPDG AELPFTIDK

A/INDONESIA Optimized M1 (SEQ ID 48)

GGTACCGGATCCGCCACCATGTCCCTGCTGACCGAGGTGGAGACCTACGTGCTGTCCATC
ATCCCCTCCGGTCCTCTGAAGGCTGAGATCGCTCAGAAGCTCGAGGACGTTTTCGCTGGC
AAGAACACCGACCTCGAGGCTCTGATGGAGTGGCTCAAGACCCGTCCCATCCTGTCCCCC
CTGACCAAGGGTATCCTGGGTTTCGTGTTCACCCTGACCGTGCCCTCCGAGCGTGGTCTG
CAGCGTCGTCGTTTCGTGCAGAACGCTCTGAACGGTAACGGTGACCCCAACAACATGGAC
CGTGCTGTGAAGCTGTACAAGAAGCTGAAGCGCGAGATCACCTTCCACGGTGCTAAGGAG
GTGTCCCTGTCCTACTCCACCGGTGCTCTGGCTAGCTGCATGGGCCTGATCTACAACCGT
ATGGGCACCGTGACCACCGAGGTGGCCTTCGGTCTGGTCTGCGCTACCTGCGAGCAGATC
GCTGACTCCCAGCACCGTTCCCACCGTCAGATGGCTACCATCACCAACCCCCTGATCCGT
CACGAGAACCGTATGGTGCTGGCTTCCACCACCGCTAAGGCTATGGAGCAGATGGCTGGT
TCCTCCGAGCAGGCTGCTGAGGCCATGGAGGTGGCCAACCAGGCTCGTCAGATGGTGCAG
GCTATGCGTACCATCGGCACCCACCCCAACTCCTCCGCTGGTCTGCGTGACAACCTGCTC
GAGAACCTGCAGGCTTACCAGAAGCGTATGGGAGTCCAGATGCAGCGCTTCAAGTAATGA
AAGCTTGAGCTC

A/INDONESIA M1 Protein sequence (SEQ ID 49)

MSLLTEVETY VLSIIPSGPL KAEIAQKLED VFAGKNTDLE ALMEWLKTRP
ILSPLTKGIL GFVFTLTVPS ERGLQRRRFV QNALNGNGDP NNMDRAVKLY
KKLKREITFH GAKEVSLSYS TGALASCMGL IYNRMGTVTT EVAFGLVCAT
CEQIADSQHR SHRQMATITN PLIRHENRMV LASTTAKAME QMAGSSEQAA
EAMEVANQAR QMVQAMRTIG THPNSSAGLR DNLLENLQAY QKRMGVQMQR
FK

A/Anhui/1/2005

A/Anhui Optimized HA (Start and stop codon are underlined) (SEQ ID 50)

GGTACCGGATCCCTCGAGATGGAGAAGATCGTGCTGCTGCTGGCTATCGTGTCCCTGGTG
AAGTCCGACCAGATCTGCATCGGTTACCACGCTAACAACTCCACCGAGCAGGTGGACACC
ATCATGGAGAAGAACGTCACCGTGACCCACGCTCAGGACATCCTGGAAAAGACCCACAAC
GGCAAGCTGTGCGACCTGGACGGTGTCAAGCCCCTGATCCTGCGTGACTGCTCCGTGGCT
GGTTGGCTGCTGGGTAACCCCATGTGCGACGAGTTCATCAACGTGCCCGAGTGGTCCTAC
ATCGTGGAGAAGGCTAACCCCGCTAACGACCTGTGCTACCCCGGTAACTTCAACGACTAC
GAGGAGCTGAAGCACCTGCTGTCCCGTATCAACCACTTCGAGAAGATCCAGATCATCCCC
AAGTCCTCTTGGTCCGACCACGAGGCTTCCTCCGGTGTCTCCTCCGCTTGCCCATACCAG
GGCACCCCATCTTTCTTCCGTAACGTGGTGTGGCTGATCAAGAAGAACAACACCTACCCC
ACCATCAAGCGTTCCTACAACAACACCAACCAGGAGGACCTGCTGATCCTGTGGGGTATC
CACCACTCCAACGACGCTGCCGAGCAGACCAAGCTGTACCAGAACCCCACCACCTACATC
TCCGTGGGCACCTCCACCCTGAACCAGCGTCTGGTGCCCAAGATCGCTACCCGTTCCAAG
GTGAACGGCCAGTCCGGTCGTATGGACTTCTTCTGGACCATCCTGAAGCCTAACGACGCT
ATCAACTTCGAGTCCAACGGCAACTTCATCGCTCCCGAGTACGCTTACAAGATCGTGAAG
AAGGGCGACTCCGCTATCGTCAAGTCCGAGGTGGAGTACGGTAACTGCAACACCAAGTGC
CAGACCCCCATCGGTGCTATCAACTCCTCCATGCCCTTCCACAACATCCACCCCCTGACC
ATCGGCGAGTGCCCCAAGTACGTGAAGTCCAACAAGCTGGTGCTGGCTACCGGTCTGCGT
AACTCCCCCCTGCGTGAGCGTGGTCTGTTCGGCGCTATCGCTGGTTTCATCGAGGGCGGT
TGGCAGGGCATGGTGGACGGTTGGTACGGTTACCACCACAGCAACGAGCAGGGTTCCGGT
TACGCTGCTGACAAGGAGTCCACCCAGAAGGCTATCGACGGCGTCACCAACAAGGTGAAC
TCCATCATCGACAAGATGAACACCCAGTTCGAGGCTGTGGGTCGTGAGTTCAACAACCTG
GAGCGTCGTATCGAGAACCTGAACAAGAAGATGGAGGACGGTTTCCTGGACGTGTGGACC
TACAACGCCGAGCTGCTGGTGCTGATGGAGAACGAGCGTACCCTGGACTTCCACGACTCT
AACGTGAAGAACCTGTACGACAAGGTCCGCCTGCAGCTGCGTGACAACGCTAAGGAGCTG
GGTAACGGTTGCTTCGAGTTCTACCACAAGTGCGACAACGAGTGCATGGAGTCCGTGCGT
AACGGCACCTACGACTACCCCCAGTACTCCGAGGAGGCTCGTCTGAAGCGTGAGGAGATC
TCCGGCGTGAAGCTGGAGTCCATCGGCACCTACCAGATCCTGTCCATCTACTCCACCGTG
GCTTCCTCCCTGGCTCTGGCTATCATGGTGGCTGGTCTGTCCCTGTGGATGTGCTCCAAC
GGTTCCCTGCAGTGCCGTATCTGCATCTAATAATGAGGCGCGCCAAGCTTGAGCTC

A/Anhui HA Protein sequence (SEQ ID 51)

MEKIVLLLAI VSLVKSDQIC IGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFINVP EWSYIVEKAN
PANDLCYPGN FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYQGTPSFF RNVVWLIKKN NTYPTIKRSY NNTNQEDLLI LWGIHHSNDA
AEQTKLYQNP TTYISVGTST LNQRLVPKIA TRSKVNGQSG RMDFFWTILK
PNDAINFESN GNFIAPEYAY KIVKKGDSAI VKSEVEYGNC NTKCQTPIGA
INSSMPFHNI HPLTIGECPK YVKSNKLVLA TGLRNSPLRE RGLFGAIAGF
IEGGWQGMVD GWYGYHHSNE QGSGYAADKE STQKAIDGVT NKVNSIIDKM
NTQFEAVGRE FNNLERRIEN LNKKMEDGFL DVWTYNAELL VLMENERTLD
FHDSNVKNLY DKVRLQLRDN AKELGNGCFE FYHKCDNECM ESVRNGTYDY
PQYSEEARLK REEISGVKLE SIGTYQILSI YSTVASSLAL AIMVAGLSLW
MCSNGSLQCR ICI

A/Bar headed goose/Qinghai/1A/2005

A/Qinghai Optimized HA (Start and stop codon are underlined) (SEQ ID 52)

CGGGCGCGGAGCGGCCGCATGGAGAAGATCGTGCTGCTGCTGGCTATCGTGTCTCTGGTCAAGTCCGACCAGATCTGCA
TCGGTTACCACGCTAACAACTCCACCGAGCAGGTGGACACCATCATGGAGAAGAACGTCACCGTGACCCACGCTCAGGA
CATCCTCGAAAAGACCCACAACGGCAAGCTGTGCGACCTGGACGGCGTGAAGCCCCTGATCCTGCGTGACTGCTCCGTG
GCTGGTTGGCTGCTGGGTAACCCCATGTGCGACGAGTTCCTCAACGTGCCCGAGTGGTCCTACATCGTGGAGAAGATCA
ACCCCGCTAACGACCTGTGCTACCCCGGTAACTTCAACGACTACGAGGAGCTGAAGCACCTGCTGTCCCGTATCAACCA
CTTCGAGAAGATCCAGATCATCCCCAAGTCCTCTTGGTCCGACCACGAGGCTTCCTCCGGTGTCTCCTCCGCTTGCCCA
TACCAGGGCCGTTCTTCCTTCTTCCGCAACGTGGTGTGGCTGATCAAGAAGAACAACGCCTACCCCACCATCAAGCGTT
CCTACAACAACACCAACCAGGAGGACCTGCTGGTCCTGTGGGGTATCCACCACCCCAACGACGCTGCCGAGCAGACCCG
TCTGTACCAGAACCCCACCACCTACATCTCCGTGGGCACCTCTACCCTGAACCAGCGTCTGGTGCCCAAGATCGCTACC
CGTTCCAAGGTGAACGGCCAGTCCGGTCGTATGGAGTTCTTCTGGACCATCCTGAAGCCTAACGACGCTATCAACTTCG
AGTCCAACGGCAACTTCATCGCTCCCGAGAACGCTTACAAGATCGTGAAGAAGGGCGACTCCACCATCATGAAGTCCGA
GCTGGAGTACGGCAACTGCAACACTAAGTGCCAGACCCCCATCGGTGCTATCAACTCCTCCATGCCCTTCCACAACATC
CACCCCCTGACTATCGGCGAGTGCCCCAAGTACGTGAAGTCCAACCGTCTGGTGCTGGCTACCGGTCTGCGTAACTCCC
CCCAGATCGAGACTCGTGGTCTGTTCGGCGCTATCGCTGGTTTCATCGAGGGCGGTTGGCAGGGCATGGTGGACGGTTG
GTACGGTTACCACCACTCTAACGAGCAGGGTTCCGGTTACGCTGCTGACAAGGAGTCTACCCAGAAGGCTATCGACGGC
GTCACCAACAAGGTGAACTCCATCATCGACAAGATGAACACCCAGTTCGAGGCTGTGGGTCGTGAGTTCAACAACCTCG
AACGTCGTATCGAGAACCTGAACAAGAAGATGGAGGACGGTTTCCTGGACGTGTGGACCTACAACGCCGAGCTGCTGGT
GCTGATGGAGAACGAGCGTACCCTGGACTTCCACGACTCCAACGTGAAGAACCTGTACGACAAGGTCCGCCTGCAGCTG
CGTGACAACGCTAAGGAGCTGGGTAACGGTTGCTTCGAGTTCTACCACCGTTGCGACAACGAGTGCATGGAGTCCGTGC
GTAACGGCACCTACGACTACCCCCAGTACTCCGAGGAGGCTCGTCTGAAGCGTGAGGAGATCTCCGGTGTCAAGCTCGA
ATCCATCGGAACCTACCAGATCCTGTCCATCTACTCCACCGTGGCTTCCTCCCTGGCTCTGGCTATCATGGTGGCTGGT
CTGTCCCTGTGGATGTGCTCCAACGGTTCCCTGCAGTGCCGTATCTGCATCTAATAATGAGGCGCGCCAAGCTTGTCGA

A/Qinghai **HA Protein sequence**(SEQ ID 53)

MEKIVLLLAI VSLVKSDQIC IGYHANNSTE QVDTIMEKNV TVTHAQDILE
KTHNGKLCDL DGVKPLILRD CSVAGWLLGN PMCDEFLNVP EWSYIVEKIN
PANDLCYPGN FNDYEELKHL LSRINHFEKI QIIPKSSWSD HEASSGVSSA
CPYQGRSSFF RNVVWLIKKN NAYPTIKRSY NNTNQEDLLV LWGIHHPNDA
AEQTRLYQNP TTYISVGTST LNQRLVPKIA TRSKVNGQSG RMEFFWTILK
PNDAINFESN GNFIAPENAY KIVKKGDSTI MKSELEYGNC NTKCQTPIGA
INSSMPFHNI HPLTIGECPK YVKSNRLVLA TGLRNSPQIE TRGLFGAIAG
FIEGGWQGMV DGWYGYHHSN EQGSGYAADK ESTQKAIDGV TNKVNSIIDK
MNTQFEAVGR EFNNLERRIE NLNKKMEDGF LDVWTYNAEL LVLMENERTL
DFHDSNVKNL YDKVRLQLRD NAKELGNGCF EFYHRCDNEC MESVRNGTYD
YPQYSEEARL KREEISGVKL ESIGTYQILS IYSTVASSLA LAIMVAGLSL
WMCSNGSLQC RICI

A/Qinghai Optimized NA (Start and stop codon are underlined) (SEQ ID 54)

ACCGTCCCACCATCGGGCGCGGATCCCTCGAGATGAACCCCAACCAGAAGATCATCACCATCGGCTCCATCTGCATGGT
GATCGGTATCGTGTCCCTGATGCTGCAGATCGGTAACATGATCTCCATCTGGGTGTCCCACTCCATCCAGACCGGTAAC
CAGCGTCAGGCCGAGCCCATCTCCAACACCAAGTTCCTCACCGAGAAGGCTGTGGCTTCCGTGACCCTGGCTGGTAACT
CCTCCCTGTGCCCCATCTCCGGTTGGGCTGTGTACTCCAAGGACAACTCCATCCGTATCGGTTCCCGTGGTGACGTGTT
CGTGATCCGTGAGCCCTTCATCTCCTGCTCCCACCTCGAATGCCGTACCTTCTTCCTGACCCAGGGTGCTCTGCTGAAC
GACAAGCACTCCAACGGCACCGTGAAGGACCGTTCCCCCCACCGTACCCTGATGTCCTGCCCCGTGGGCGAGGCTCCCT
CCCCCTACAACTCCCGTTTCGAGTCCGTGGCTTGGTCCGCTTCCGCTTGCCACGACGGCACCTCTTGGCTGACCATCGG
TATCTCCGGTCCCGACAACGGTGCTGTGGCTGTGCTGAAGTACAACGGCATCATCACCGACACCATCAAGTCCTGGCGT
AACAACATCCTGCGTACCCAAGAGTCCGAGTGCGCTTGCGTGAACGGTTCCTGCTTCACCGTGATGACCGACGGTCCCT
CCAACGGCCAGGCTTCCTACAAGATCTTCAAGATGGAGAAGGGCAAGGTGGTGAAGTCCGTGGAGCTGGACGCTCCCAA
CTACCACTACGAGGAGTGCTCTTGCTACCCCGACGCTGGCGAGATCACCTGCGTGTGCCGTGACAACTGGCACGGTTCC
AACCGTCCCTGGGTGTCCTTCAACCAGAACCTCGAATACCAGATCGGTTACATCTGCTCCGGCGTGTTCGGTGACAACC
CCCGTCCCAACGACGGAACCGGTTCCTGCGGTCCCGTGTCCCCCAACGGTGCTTACGGTGTCAAGGGCTTCTCCTTCAA
GTACGGTAACGGTGTCTGGATCGGTCGTACCAAGTCCACCAACTCCCGCTCCGGTTTCGAGATGATCTGGGACCCCAAC
GGTTGGACCGGCACCGACTCTTCCTTCTCCGTGAAGCAGGACATCGTGGCTATCACCGACTGGTCCGGTTACTCCGGTT
CCTTCGTGCAGCACCCCGAGCTGACCGGTCTGGACTGTATCCGTCCCTGCTTCTGGGTGGAGCTGATCCGTGGTCGTCC
CAAGGAGTCCACCATCTGGACCTCCGGCTCCTCCATCTCTTTCTGCGGTGTGAACTCCGACACCGTGTCCTGGTCCTGG
CCCGACGGTGCCGAGCTGCCCTTCACCATCGACAAGTAATAATGAATCGATTTGTCGAGAAGTACTAGAGGATCATAAT
Protein sequence:

A/Qinghai NA Protein sequence(SEQ ID 55)

MNPNQKIITI GSICMVIGIV SLMLQIGNMI SIWVSHSIQT GNQRQAEPIS
NTKFLTEKAV ASVTLAGNSS LCPISGWAVY SKDNSIRIGS RGDVFVIREP
FISCSHLECR TFFLTQGALL NDKHSNGTVK DRSPHRTLMS CPVGEAPSPY
NSRFESVAWS ASACHDGTSW LTIGISGPDN GAVAVLKYNG IITDTIKSWR
NNILRTQESE CACVNGSCFT VMTDGPSNGQ ASYKIFKMEK GKVVKSVELD
APNYHYEECS CYPDAGEITC VCRDNWHGSN RPWVSFNQNL EYQIGYICSG
VFGDNPRPND GTGSCGPVSP NGAYGVKGFS FKYGNGVWIG RTKSTNSRSG
FEMIWDPNGW TGTDSSFSVK QDIVAITDWS GYSGSFVQHP ELTGLDCIRP
CFWVELIRGR PKESTIWTSG SSISFCGVNS DTVSWSWPDG AELPFTIDK

The following references are incorporated herein by reference:

Berglund, P., Fleeton, M. N., Smerdou, C., and Liljestrom, P. (1999). Immunization with recombinant Semliki Forest virus induces protection against influenza challenge in mice. Vaccine 17, 497-507.

Cox, J. C., and Coulter, A. R.(1997). Adjuvants--a classification and review of their modes of action. Vaccine 15, 248-256.

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Peiris, J. S., Guan, Y., Markwell, D., Ghose, P., Webster, R. G., and Shortridge, K. F. (2001). Cocirculation of avian H9N2 and contemporary "human" H3N2 influenza A viruses in pigs in southwestern China: potential for genetic reassortment? J. Virol. 75, 9679-9686.

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Treanor, J. J., Betts, R. F., Smith, G. E., Anderson, E. L., Hackett, C. S., Wilkinson, B. E., Belshe, R. B., and Powers, D. C. (1996). Evaluation of a recombinant hemagglutinin expressed in insect cells as an influenza vaccine in young and elderly adults. J. Infect. Dis. 173, 1467-1470.

Tsuji, M., et al. (1998). Recombinant Sindbis viruses expressing a cytotoxic T-lymphocyte epitope of a malaria parasite or of influenza virus elicit protection against the corresponding pathogen in mice. J. Virol. 72, 6907-6910.

Ulmer, J. B., et al. (1993). Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259, 1745-1749.

Ulmer, J. B., et al. (1998). Protective CD4+ and CD8+T cells against influenza virus induced by vaccination with nucleoprotein DNA. J. Virol. 72, 5648-5653.

Watanabe, T., Watanabe, S., Neumann, G., and Kawaoka, Y. (2002) Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. J. Virol. 76, 767-773.

Zhou, X., et al. (1995). Generation of cytotoxic and humoral immune responses by non-replicative recombinant Semliki Forest virus. Proc. Natl. Acad. Sci. USA 92, 3009-3013.

Other Embodiments

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims:

Claims:

1. A virus like particle (VLP) comprising an influenza virus M1 protein and influenza virus H5 and N1 hemagglutinin and neuraminidase proteins.

2. The VLP of claim 1, wherein said M1 protein is derived from a different influenza virus strain as compared to the H5 and N1 proteins.

3. The VLP of claim 1, wherein said H5 or N1 are from a H5N1 clade 1 influenza virus.

4. The VLP of claim 1, wherein said H5 and N1 are from a H5N1 clade 2 influenza virus.

5. The VLP of claim 3, wherein said H5 and N1 proteins comprise SEQ ID NOS: 43 and 46, respectively, or a sequence comprising at least 90% sequence identity to said sequences.

6. The VLP of claim 4, wherein said H5 and N1 proteins comprise SEQ ID NOS: 50 and 54, respectively, or a sequence comprising at least 90% sequence identity to said sequences.

7. The VLP of claim 1, wherein said H5 and N1 are from an influenza virus which was isolated from an infected animal.

8. The VLP of claim 7, wherein said infected animal is a human.

9. The VLP of claim 1, wherein the VLP is expressed from a eukaryotic cell comprising one or more nucleic acids encoding influenza H5 and N1 proteins and an influenza M1 protein under conditions that permit the formation of VLPs.

10. The VLP of claim 9, wherein said eukaryotic cell is selected from the group consisting of yeast, insect, amphibian, avian and mammalian cells.

11. The VLP of claim 10, wherein said eukaryotic cell is an insect cell.

12. The VLP of claim 11, wherein said insect cell is Sf9.

13. The VLP of claim 1, wherein said VLP elicits neutralizing antibodies in a human or animal that are protective against influenza infection when administered to said human or animal.

14. An immunogenic composition comprising an effective dose of a VLP of any one of claims 1-13.

15. The composition of claim 14, wherein said composition comprises an adjuvant.

16. A vaccine comprising an effective dose of a VLP of any one of claims 1-13.

17. The vaccine of claim 16, wherein said vaccine comprises at least a second VLP which comprises HA and NA from different influenza strains.

18. The vaccine of claims 16 or 17, wherein said vaccine comprises an adjuvant.

19. The immunogenic composition or vaccine of claim 15 or 18 wherein said adjuvant comprises Novasomes®.

20. A method of inducing substantial immunity to influenza virus infection in an animal, comprising administering at least one effective dose a vaccine of any one of claims 16-18.

21. A method of claim 20, wherein the vaccine is administered to an animal orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

22. A method of claim 20 or 21, wherein the animal is a human.

23. The use of a VLP of any one of claims 1-13, for the preparation of a vaccine for an animal, wherein the vaccine induces substantial immunity to influenza virus infection in said animal.

24. The use of claim 23, wherein the vaccine is administered to the animal orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

25. A method of making a VLP of any one of claims 1-13, comprising expressing said M1, HA and NA proteins in a eukaryotic cell.

26. The method of claim 25, wherein said eukaryotic cell is selected from the group consisting of yeast, insect, amphibian, avian and mammalian cells.

27. The method of claim 26, wherein said eukaryotic cell is an insect cell.

28. The method of claim 27, wherein said insect cell is Sf9.

29. An insect cell expression vector encoding at least one influenza H5 or N1 protein.

30. A vaccine comprising an influenza VLP, wherein said VLP comprises influenza M1, HA and NA proteins, wherein said vaccine induces substantial immunity to influenza virus infection in a human.

31. A vaccine comprising an influenza VLP, wherein said VLP consists essentially of influenza M1, HA and NA proteins, wherein said vaccine induces substantial immunity to influenza virus infection in a human.

32. A vaccine comprising an influenza VLP, wherein said VLP comprises influenza proteins selected from the group consisting of influenza M1, HA and NA proteins, wherein said vaccine induces substantial immunity to influenza virus infection in a human.

33. A method of inducing substantial immunity to influenza virus infection in a human, comprising administering at least one effective dose a vaccine of any one of claims 30-32.

34. A method of claim 33, wherein the vaccine is administered orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

35. The use of an influenza VLP, wherein said VLP comprises influenza M1, HA and NA proteins, for the preparation of a vaccine, wherein the vaccine induces substantial immunity to influenza virus infection in a human.

36. The use of an influenza VLP, wherein said VLP consists essentially of influenza M1, HA and NA proteins, for the preparation of a vaccine, wherein the vaccine induces substantial immunity to influenza virus infection in a human.

37. The use of an influenza VLP, wherein said VLP comprises influenza proteins selected from the group consisting of influenza M1, HA and NA proteins, for the preparation of a vaccine, wherein the vaccine induces substantial immunity to influenza virus infection in a human.

38. The use of any one of claims 35-37, wherein the vaccine is administered to a human orally, intradermally, intranasally, intramusclarly, intraperitoneally, intravenously, or subcutaneously.

39. A vaccine of any one of claims 16-19 and 30-32, wherein said vaccine has been treated to inactivate baculovirus.

40. The vaccine of claim 40, wherein said inactivation treatment comprises incubating a sample comprising VLPs in about a 0.2% of β-propyl lactone (BPL) for about 3 hours at about 25 °C.

**ATG**AATCCAAATCAAAAGATAATAGCACTTGGCTCTGTTTCTATAACTATTGCGACAATATG
TTTACTCATGCAGATTGCCATCTTAGCAACGACTATGACACTACATTTCAATGAATGTACCA
ACCCATCGAACAATCAAGCAGTGCCATGTGAACCAATCATAATAGAAAGGAACATAACAGAG
ATAGTGCATTTGAATAATACTACCATAGAGAAGGAAAGTTGTCCTAAAGTAGCAGAATACAA
GAATTGGTCAAAACCGCAATGTCAAATTACAGGGTTCGCCCCTTTCTCCAAGGACAACTCAA
TTAGGCTTTCTGCAGGCGGGGATATTTGGGTGACAAGAGAACCTTATGTATCGTGCGGTCTT
GGTAAATGTTACCAATTTGCACTTGGGCAGGGAACCACTTTGAACAACAAACACTCAAATGG
CACAATACATGATAGGAGTCCCCATAGAACCCTTTTAATGAACGAGTTGGGTGTTCCATTTC
ATTTGGGAACCAAACAAGTGTGCATAGCATGGTCCAGCTCAAGCTGCCATGATGGGAAGGCA
TGGTTACATGTTTGTGTCACTGGGGATGATAGAAATGCGACTGCTAGCATCATTTATGATGG
GATGCTTACCGACAGTATTGGTTCATGGTCTAAGAACATCCTCAGAACTCAGGAGTCAGAAT
GCGTTTGCATCAATGGAACTTGTACAGTAGTAATGACTGATGGAAGTGCATCAGGAAGGGCT
GATACTAAAATACTATTCATTAGAGAAGGGAAAATTGTCCACATTGGTCCACTGTCAGGAAG
TGCTCAGCATGTGGAGGAATGCTCCTGTTACCCCCGGTATCCAGAAGTTAGATGTGTTTGCA
GAGACAATTGGAAGGGCTCCAATAGACCCGTGCTATATATAAATGTGGCAGATTATAGTGTT
GATTCTAGTTATGTGTGCTCAGGACTTGTTGGCGACACACCAAGAAATGACGATAGCTCCAG
CAGCAGTAACTGCAGGGATCCTAATAACGAGAGAGGGGGCCCAGGAGTGAAAGGGTGGGCCT
TTGACAATGGAAATGATGTTTGGATGGGACGAACAATCAAGAAAGATTCGCGCTCTGGTTAT
GAGACTTTCAGGGTCGTTGGTGGTTGGACTACGGCTAATTCCAAGTCACAAATAAATAGGCA
AGTCATAGTTGACAGTGATAACTGGTCTGGGTATTCTGGTATATTCTCTGTTGAAGGAAAAA
CCTGCATCAACAGGTGTTTTTATGTGGAGTTGATAAGAGGGAGACCACAGGAGACCAGAGTA
TGGTGGACTTCAAATAGCATCATTGTATTTTGTGGAACTTCAGGTACCTATGGAACAGGCTC
ATGGCCCGATGGAGCGAATATCAATTTCATGTCTATA**TAA**

FIGURE 1

**ATG**GAAACAATATCACTAATAACTATACTACTAGTAGTAACAGCAAGCAATGCAGATAAAAT
CTGCATCGGCCACCAGTCAACAAACTCCACAGAAACTGTGGACACGCTAACAGAAACCAATG
TTCCTGTGACACATGCCAAAGAATTGCTCCACACAGAGCATAATGGAATGCTGTGTGCAACA
AGCCTGGGACATCCCCTCATTCTAGACACATGCACTATTGAAGGACTAGTCTATGGCAACCC
TTCTTGTGACCTGCTGTTGGGAGGAAGAGAATGGTCCTACATCGTCGAAAGATCATCAGCTG
TAAATGGAACGTGTTACCCTGGGAATGTAGAAAACCTAGAGGAACTCAGGACACTTTTTAGT
TCCGCTAGTTCCTACCAAAGAATCCAAATCTTCCCAGACACAACCTGGAATGTGACTTACAC
TGGAACAAGCAGAGCATGTTCAGGTTCATTCTACAGGAGTATGAGATGGCTGACTCAAAAGA
GCGGTTTTTACCCTGTTCAAGACGCCCAATACACAAATAACAGGGGAAAGAGCATTCTTTTC
GTGTGGGGCATACATCACCCACCCACCTATACCGAGCAAACAAATTTGTACATAAGAAACGA
CACAACAACAAGCGTGACAACAGAAGATTTGAATAGGACCTTCAAACCAGTGATAGGGCCAA
GGCCCCTTGTCAATGGTCTGCAGGGAAGAATTGATTATTATTGGTCGGTACTAAAACCAGGC
CAAACATTGCGAGTACGATCCAATGGGAATCTAATTGCTCCATGGTATGGACACGTTCTTTC
AGGAGGGAGCCATGGAAGAATCCTGAAGACTGATTTAAAAGGTGGTAATTGTGTAGTGCAAT
GTCAGACTGAAAAAGGTGGCTTAAACAGTACATTGCCATTCCACAATATCAGTAAATATGCA
TTTGGAACCTGCCCCAAATATGTAAGAGTTAATAGTCTCAAACTGGCAGTCGGTCTGAGGAA
CGTGCCTGCTAGATCAAGTAGAGGACTATTTGGAGCCATAGCTGGATTCATAGAAGGAGGTT
GGCCAGGACTAGTCGCTGGCTGGTATGGTTTCCAGCATTCAAATGATCAAGGGGTTGGTATG
GCTGCAGATAGGGATTCAACTCAAAAGGCAATTGATAAAATAACATCCAAGGTGAATAATAT
AGTCGACAAGATGAACAAGCAATATGAAATAATTGATCATGAATTCAGTGAGGTTGAAACTA
GACTCAATATGATCAATAATAAGATTGATGACCAAATACAAGACGTATGGGCATATAATGCA
GAATTGCTAGTACTACTTGAAAATCAAAAAACACTCGATGAGCATGATGCGAACGTGAACAA
TCTATATAACAAGGTGAAGAGGGCACTGGGCTCCAATGCTATGGAAGATGGGAAAGGCTGTT
TCGAGCTATACCATAAATGTGATGATCAGTGCATGGAAACAATTCGGAACGGGACCTATAAT
AGGAGAAAGTATAGAGAGGAATCAAGACTAGAAAGGCAGAAAATAGAGGGGGTTAAGCTGGA
ATCTGAGGGAACTTACAAAATCCTCACCATTTATTCGACTGTCGCCTCATCTCTTGTGCTTG
CAATGGGGTTTGCTGCCTTCCTGTTCTGGGCCATGTCCAATGGATCTTGCAGATGCAACATT
TGTATA**TAA**

**FIGURE 2**

**ATG**AGTCTTCTAACCGAGGTCGAAACGTACGTTCTCTCTATCATCCCATCAGGCCCCCTCAA
AGCCGAGATCGCGCAGAGACTTGAGGATGTTTTTGCAGGGAAGAACACAGATCTTGAGGCTC
TCATGGAATGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAGGGGATTTTAGGGTTT
GTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGATTTGTCCAAAATGC
CCTAAATGGGAATGGAGACCCAAACAACATGGACAGGGCAGTTAAACTATACAAGAAGCTGA
AGAGGGAAATGACATTCCATGGAGCAAAGGAAGTTGCACTCAGTTACTCAACTGGTGCGCTT
GCCAGTTGCATGGGTCTCATATACAACCGGATGGGAACAGTGACCACAGAAGTGGCTCTTGG
CCTAGTATGTGCCACTTGTGAACAGATTGCTGATGCCCAACATCGGTCCCACAGGCAGATGG
CGACTACCACCAACCCACTAATCAGGCATGAGAACAGAATGGTACTAGCCAGCACTACGGCT
AAGGCCATGGAGCAGATGGCTGGATCAAGTGAGCAGGCAGCAGAAGCCATGGAAGTCGCAAG
TCAGGCTAGGCAAATGGTGCAGGCTATGAGGACAATTGGGACTCACCCTAGTTCCAGTGCAG
GTCTAAAAGATGATCTTATTGAAAATTTGCAGGCTTACCAGAAACGGATGGGAGTGCAAATG
CAGAGATTCAAG**TGA**

**FIGURE 3**

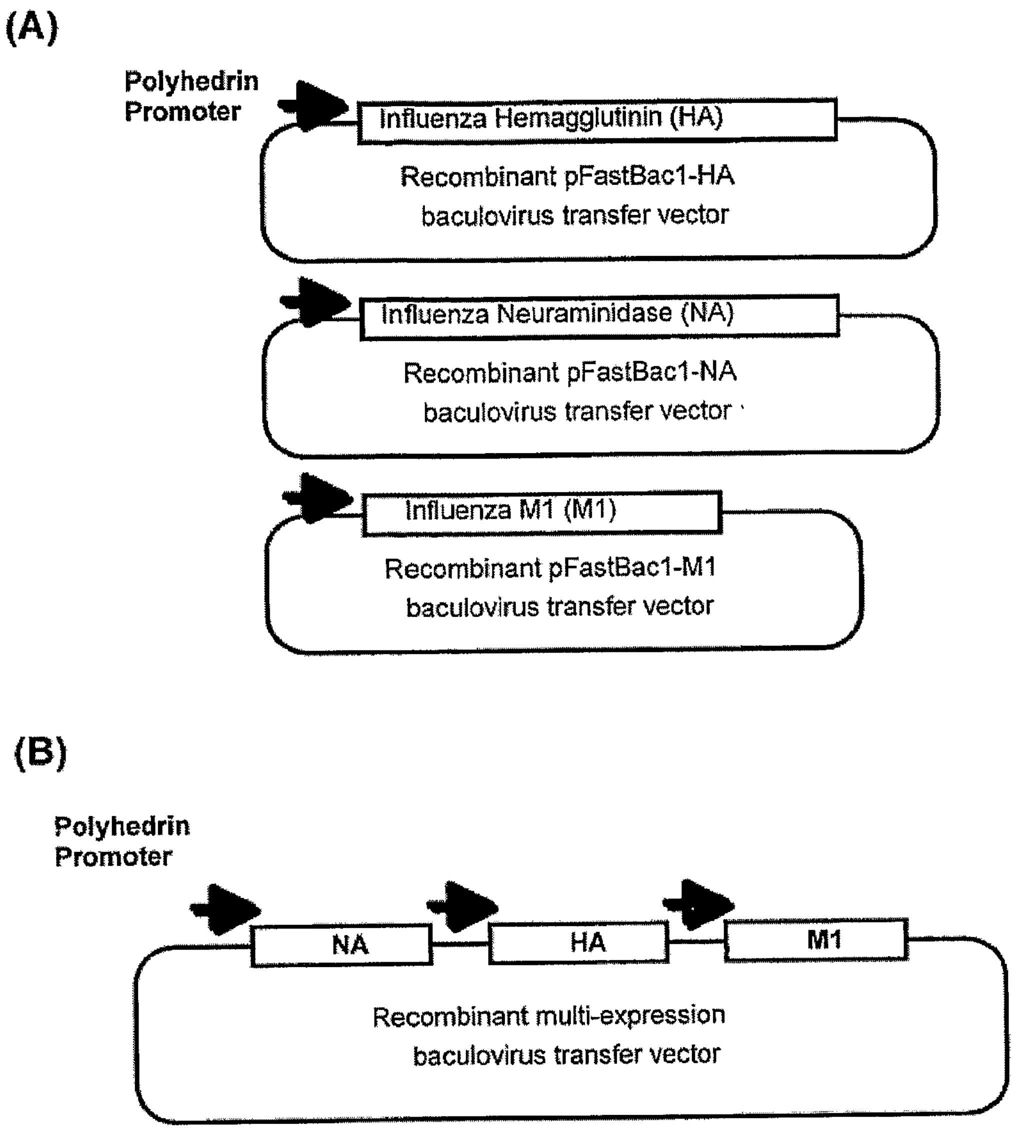

(A)
Polyhedrin Promoter
Influenza Hemagglutinin (HA)
Recombinant pFastBac1-HA baculovirus transfer vector
Influenza Neuraminidase (NA)
Recombinant pFastBac1-NA baculovirus transfer vector
Influenza M1 (M1)
Recombinant pFastBac1-M1 baculovirus transfer vector
(B)
Polyhedrin Promoter
NA
HA
M1
Recombinant multi-expression baculovirus transfer vector

FIGURE 4

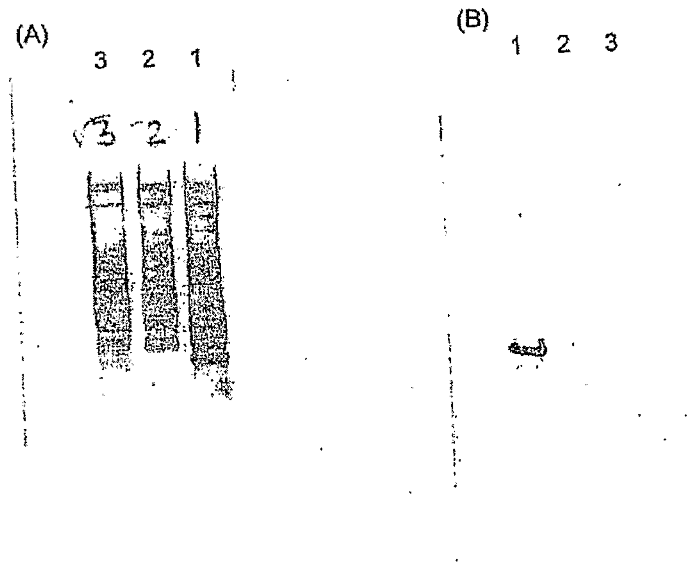



FIGURE 5

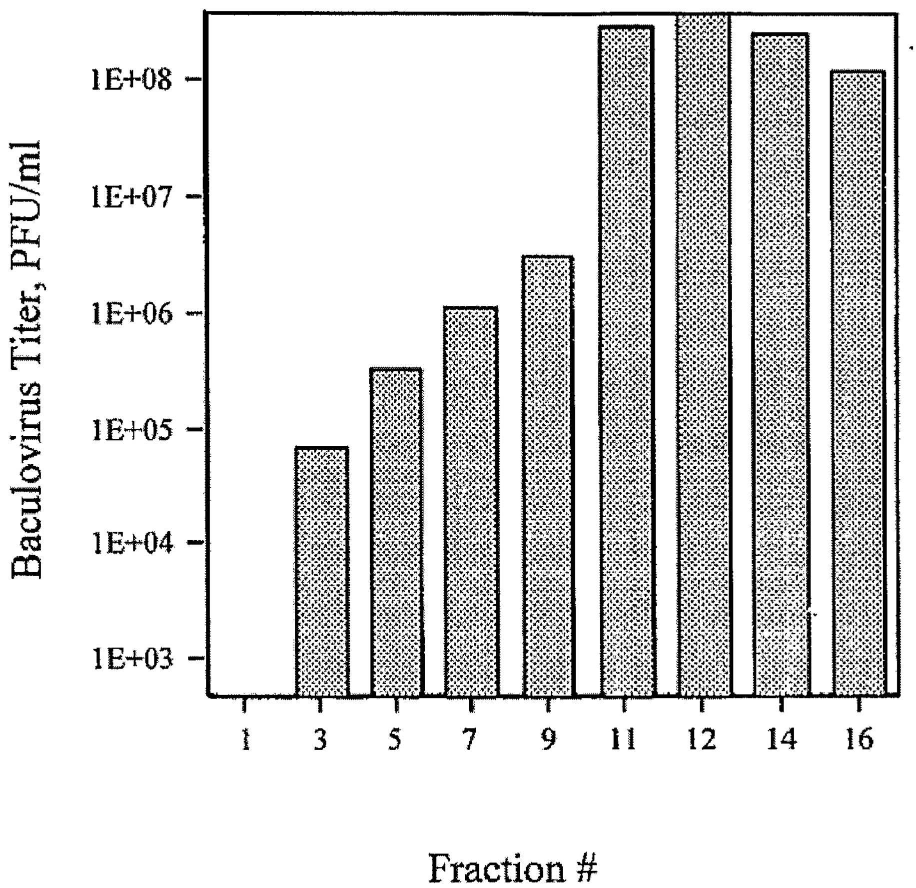

1E+08
1E+07
1E+06
1E+05
1E+04
1E+03
Baculovirus Titer, PFU/ml
1
3
5
7
9
11
12
14
16
Fraction #

FIGURE 6

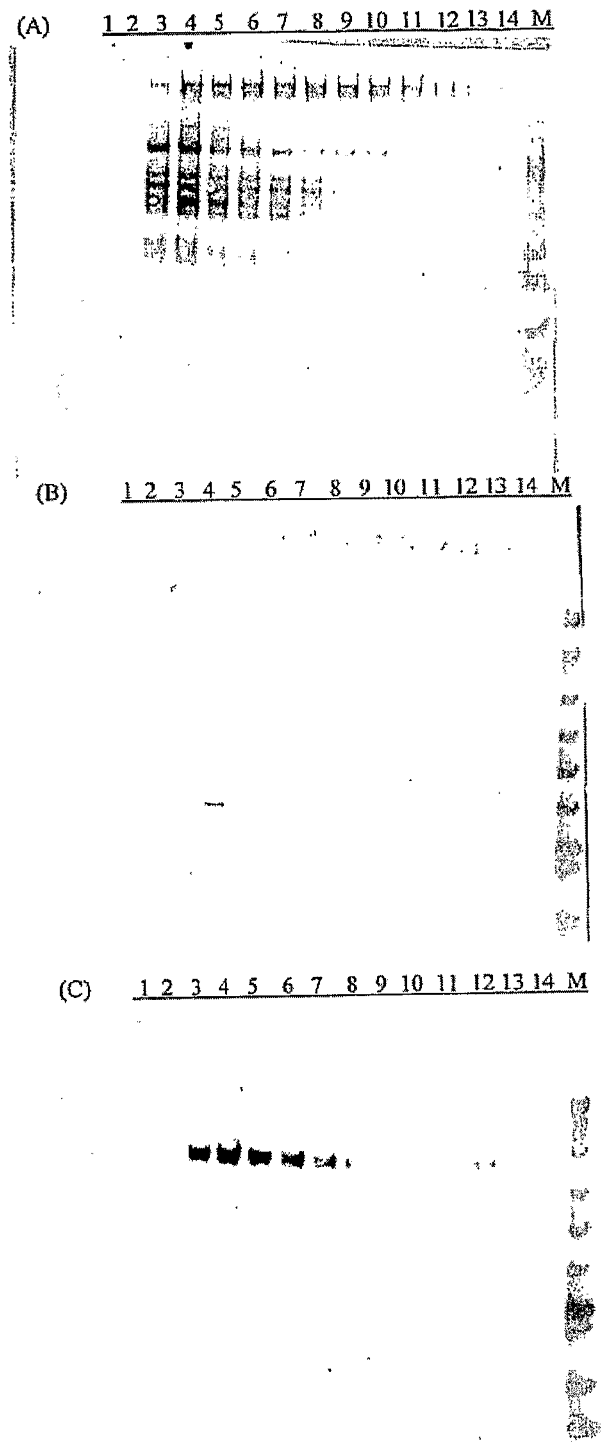

(A)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
(B)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 M
(C)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 M

**FIGURE 7**

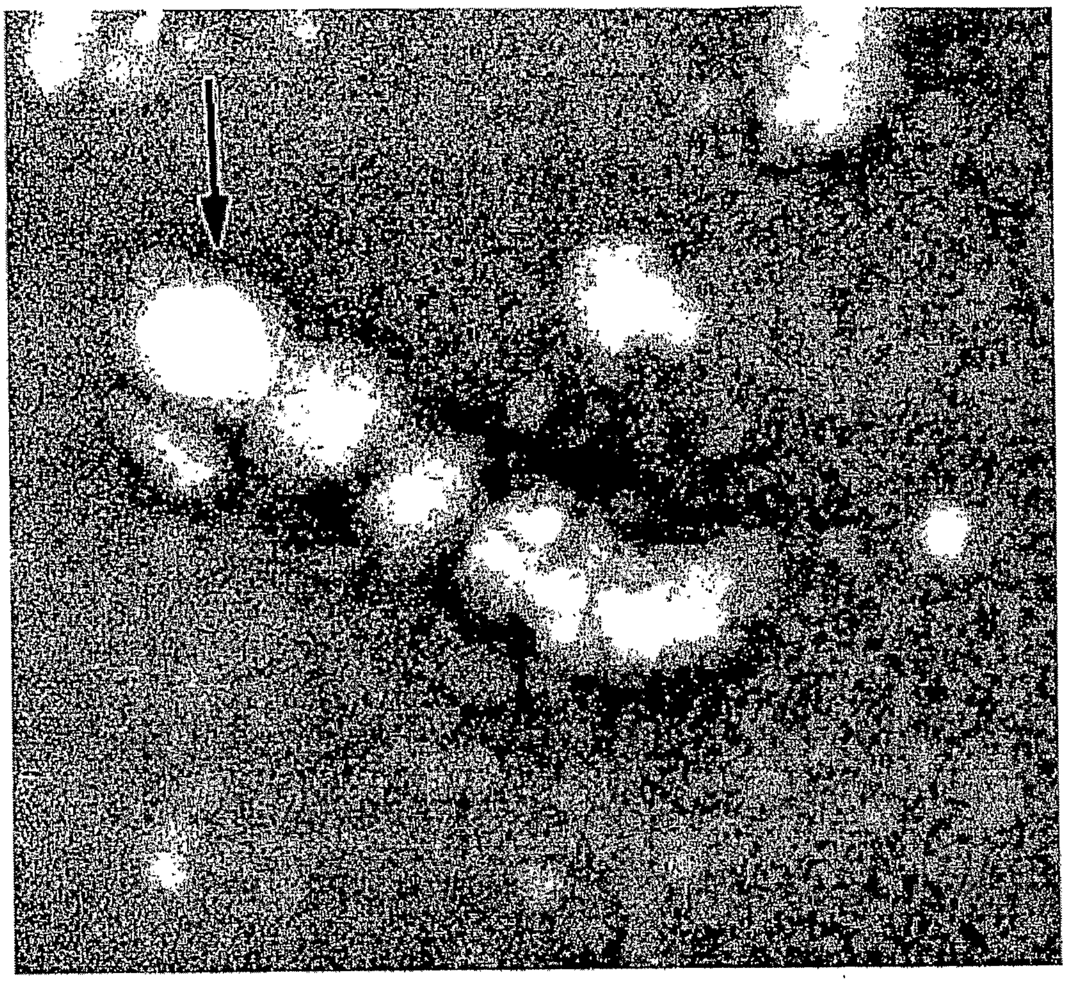


FIGURE 8

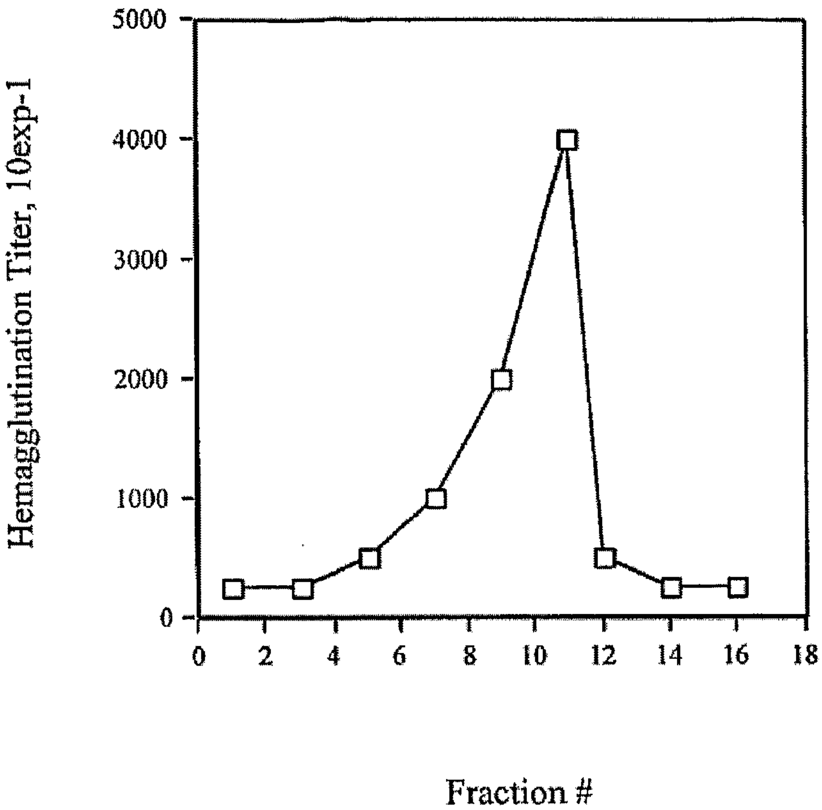

5000
4000
3000
2000
1000
0
Hemagglutination Titer, 10exp-1
0
2
4
6
8
10
12
14
16
18
Fraction #

FIGURE 9

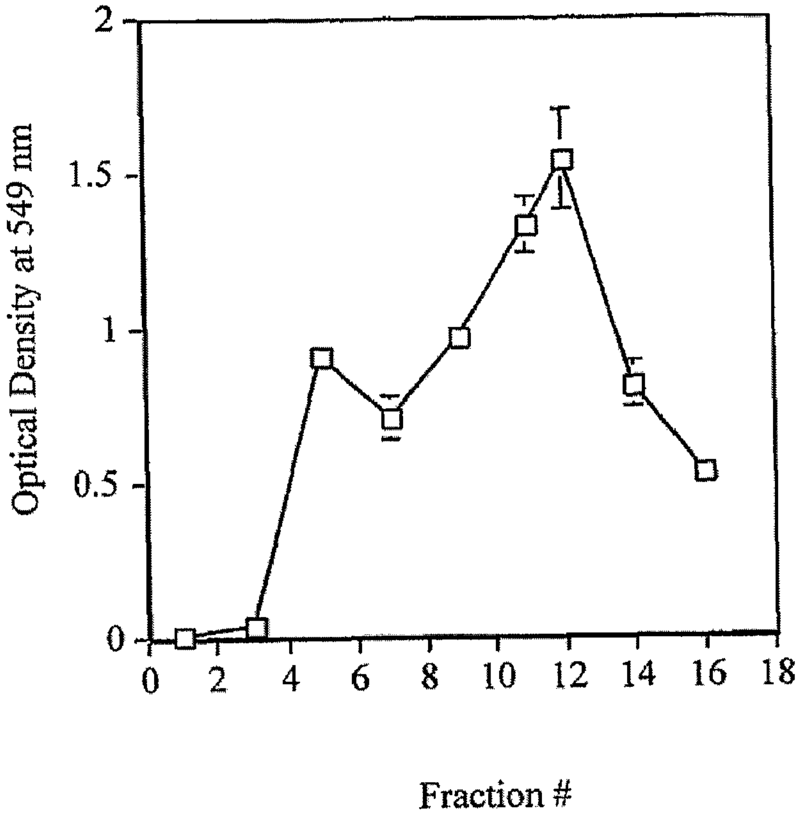

Optical Density at 549 nm
2
1.5
1
0.5
0
0
2
4
6
8
10
12
14
16
18
Fraction #

FIGURE 10

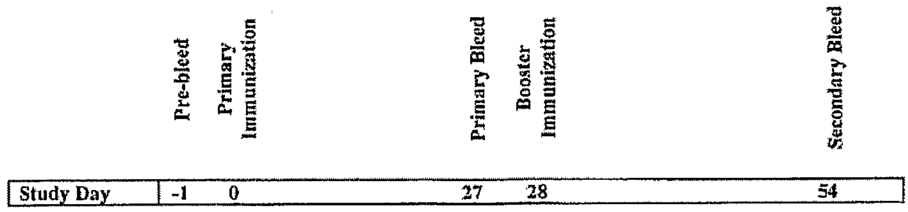

Pre-bleed
Primary Immunization
Primary Bleed
Booster Immunization
Secondary Bleed
Study Day
-1
0
27
28
54

FIGURE 11

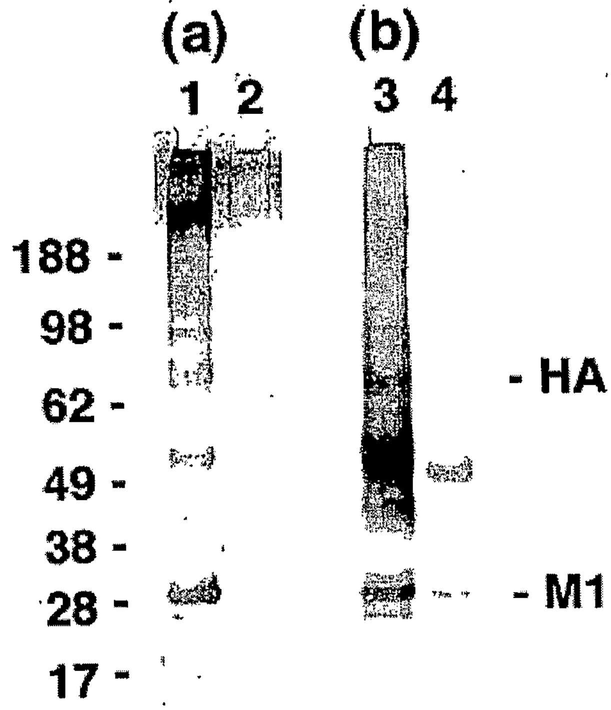



FIGURE 12

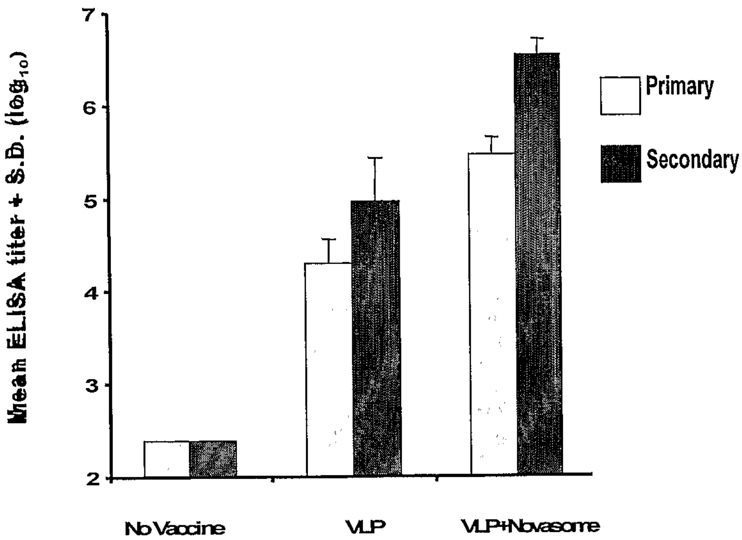
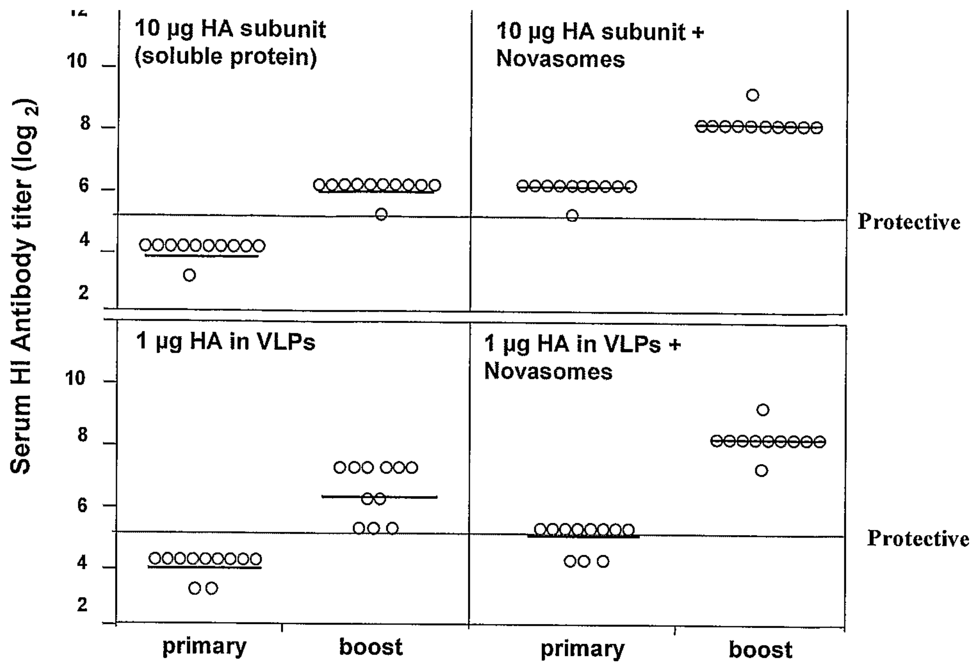

Mean ELISA titer + S.D. (log10)
7
6
5
4
3
2
Primary
Secondary
No Vaccine
VLP
VLP+Novasome

FIGURE 13



Serum HI Antibody titer (log 2)
10 μg HA subunit (soluble protein)
10 μg HA subunit + Novasomes
1 μg HA in VLPs
1 μg HA in VLPs + Novasomes
Protective
Protective
10
8
6
4
2
primary
boost
primary
boost


FIGURE 14

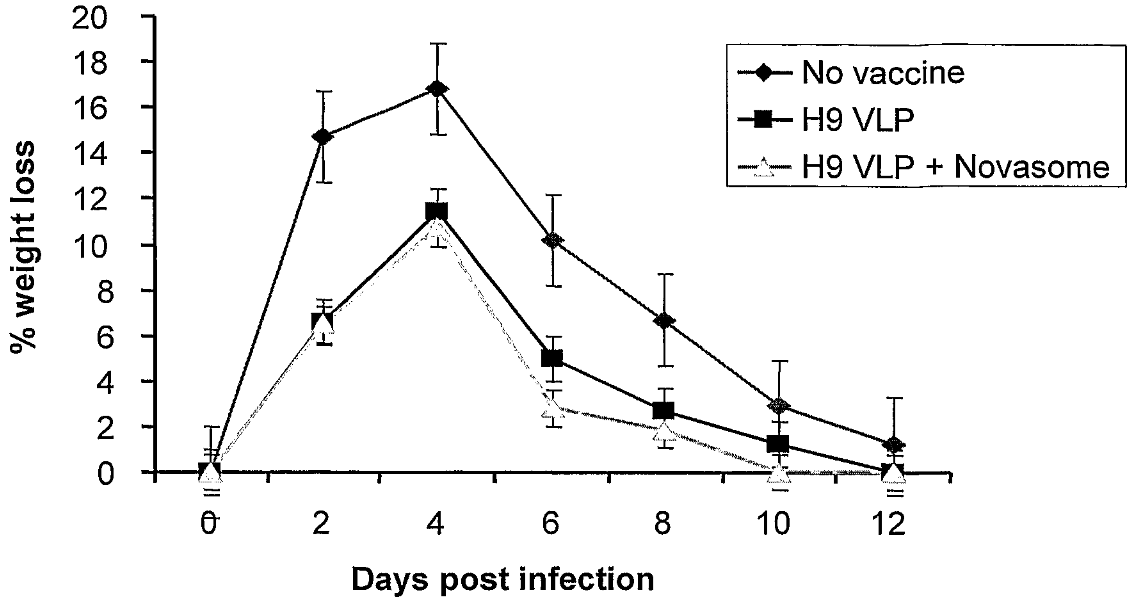

% weight loss
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
Days post infection
No vaccine
H9 VLP
H9 VLP + Novasome

FIGURE 15

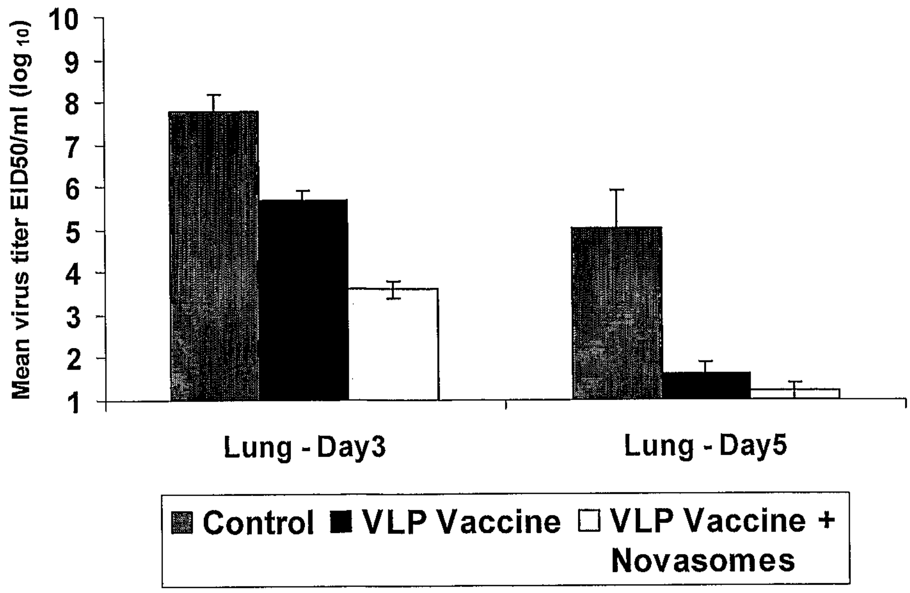

Mean virus titer EID50/ml (log 10)
10
9
8
7
6
5
4
3
2
1
Lung - Day3
Lung - Day5
Control
VLP Vaccine
VLP Vaccine + Novasomes

FIGURE 16

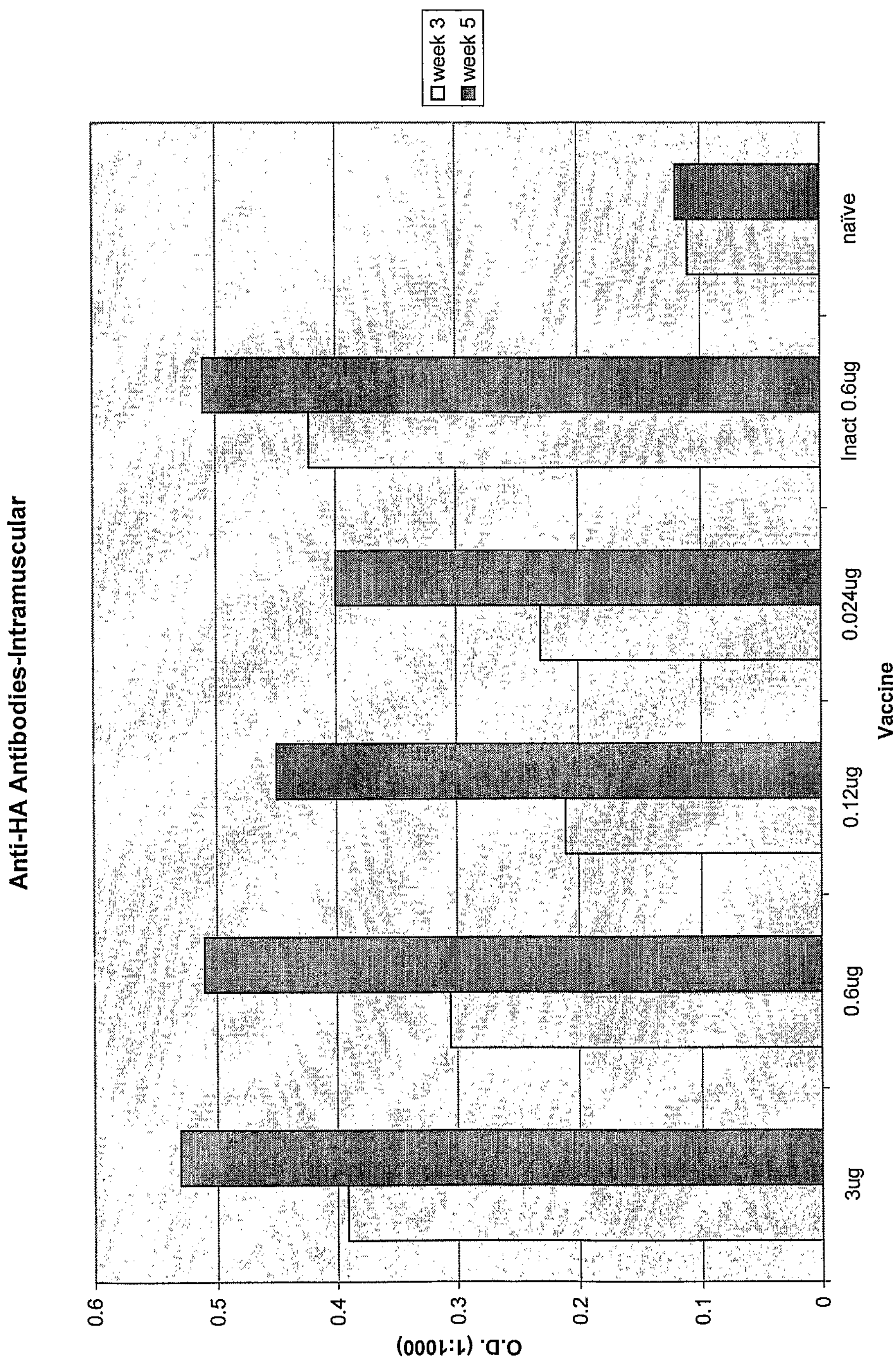

Anti-HA Antibodies-Intramuscular
week 3
week 5
O.D. (1:1000)
0
0.1
0.2
0.3
0.4
0.5
0.6
3ug
0.6ug
0.12ug
0.024ug
Inact 0.6ug
naïve
Vaccine

FIGURE 17 A

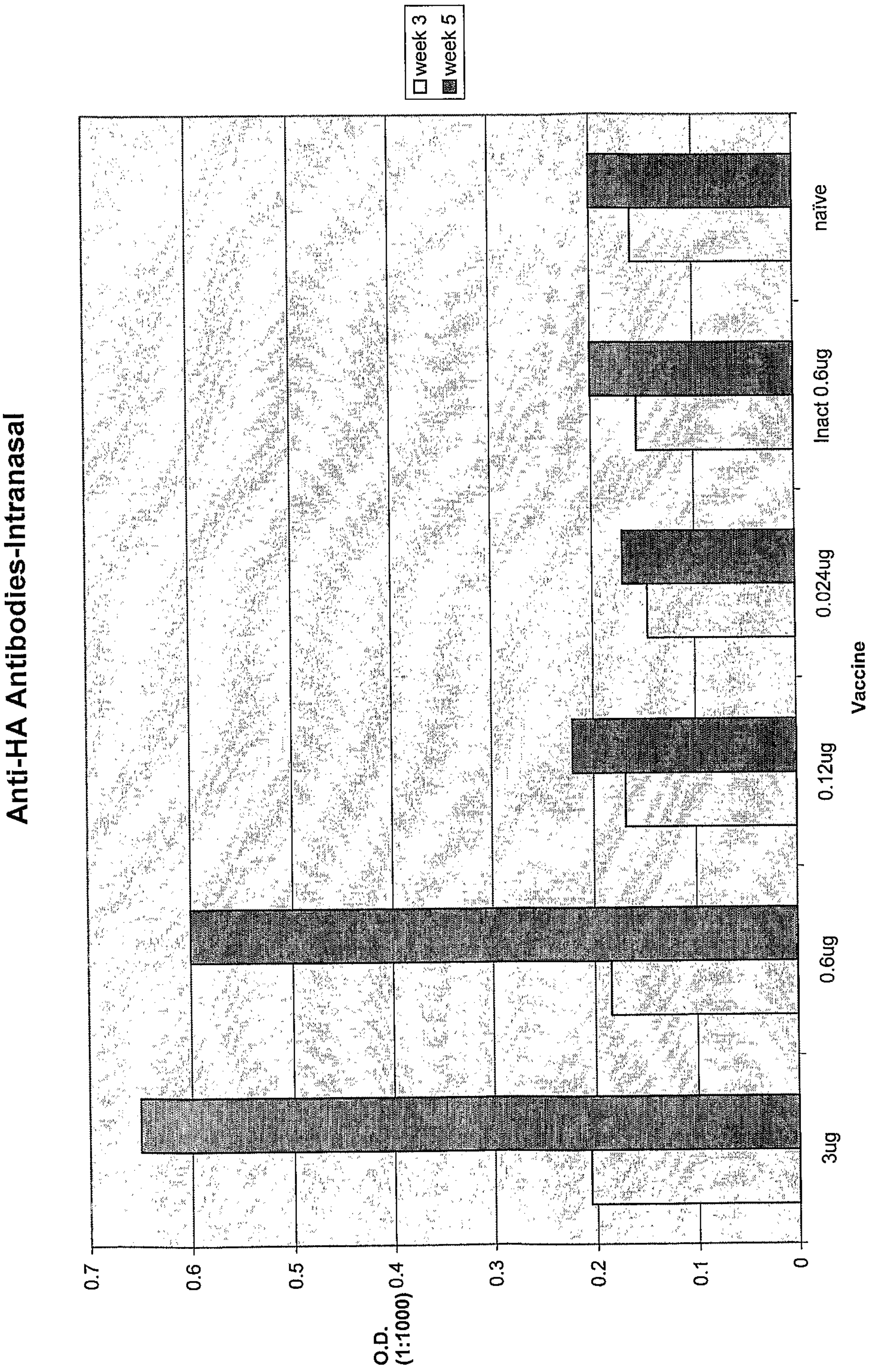

Anti-HA Antibodies-Intranasal
□ week 3
▩ week 5
O.D. (1:1000)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
3ug
0.6ug
0.12ug
0.024ug
Inact 0.6ug
naïve
Vaccine

FIGURE 17 B

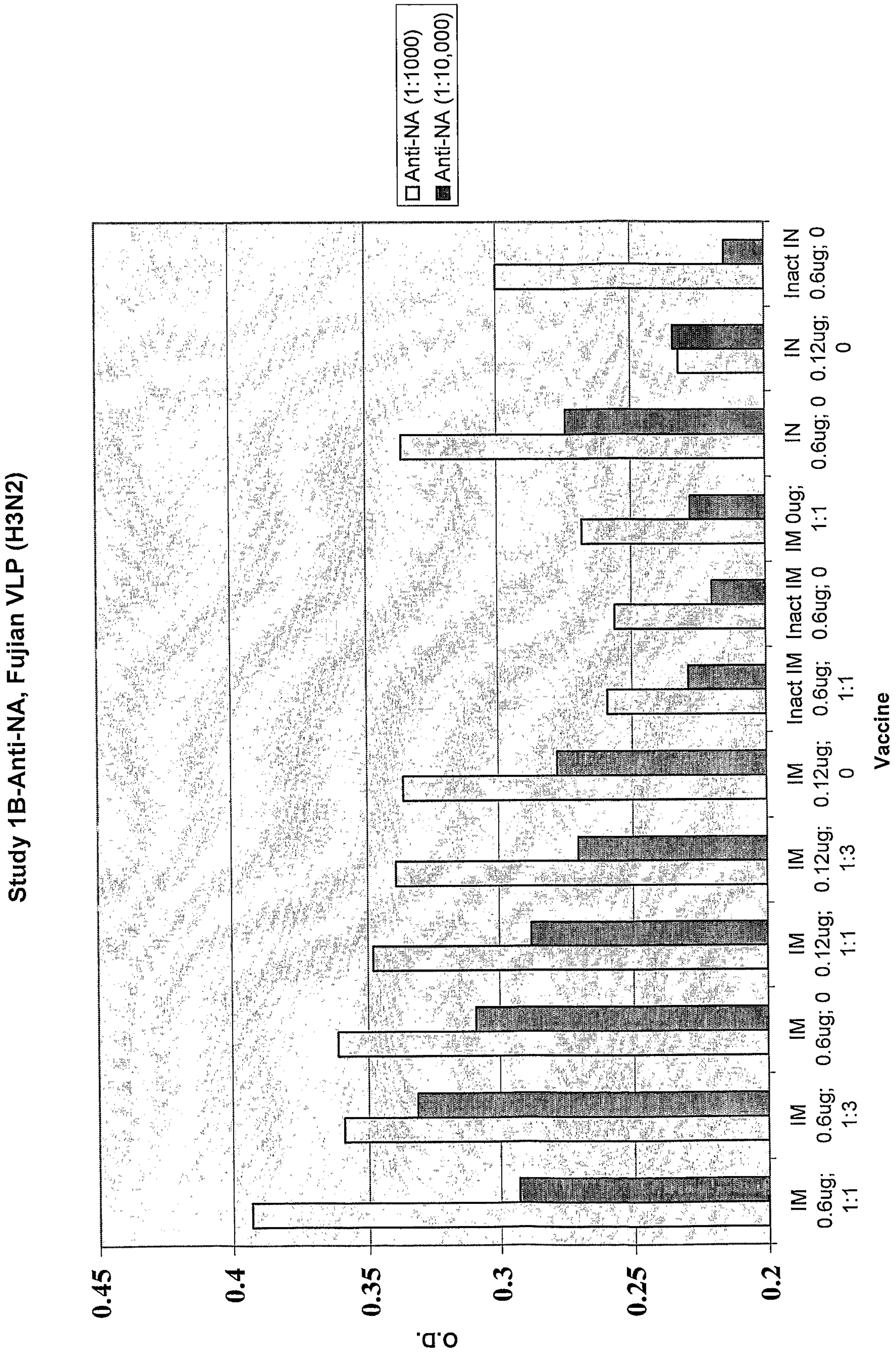

Study 1B-Anti-NA, Fujian VLP (H3N2)
Anti-NA (1:1000)
Anti-NA (1:10,000)
O.D.
0.45
0.4
0.35
0.3
0.25
0.2
IM 0.6ug; 1:1
IM 0.6ug; 1:3
IM 0.6ug; 0
IM 0.12ug; 1:1
IM 0.12ug; 1:3
IM 0.12ug; 0
Inact IM 0.6ug; 1:1
Inact IM 0.6ug; 0
IM 0ug; 1:1
IN 0.6ug; 0
IN 0.12ug; 0
Inact IN 0.6ug; 0
Vaccine

FIGURE 17 C

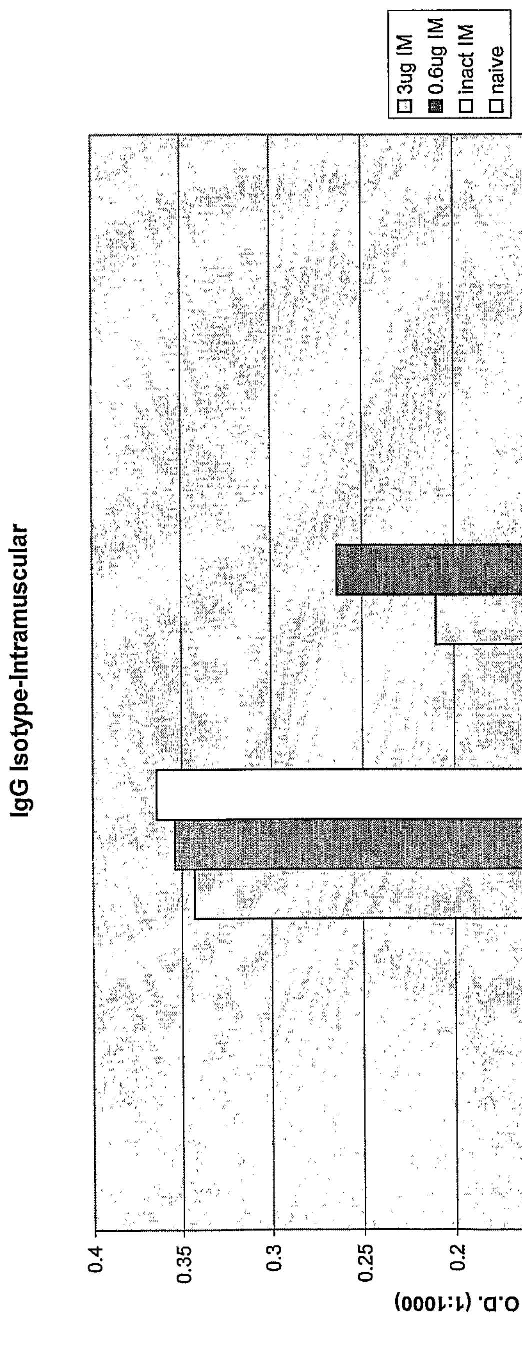

IgG Isotype-Intramuscular
3ug IM
0.6ug IM
inact IM
naive
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
O.D. (1:1000)
IgG1
IgG2a
IgG2b
IgG3
IgG Isotype

FIGURE 18 A

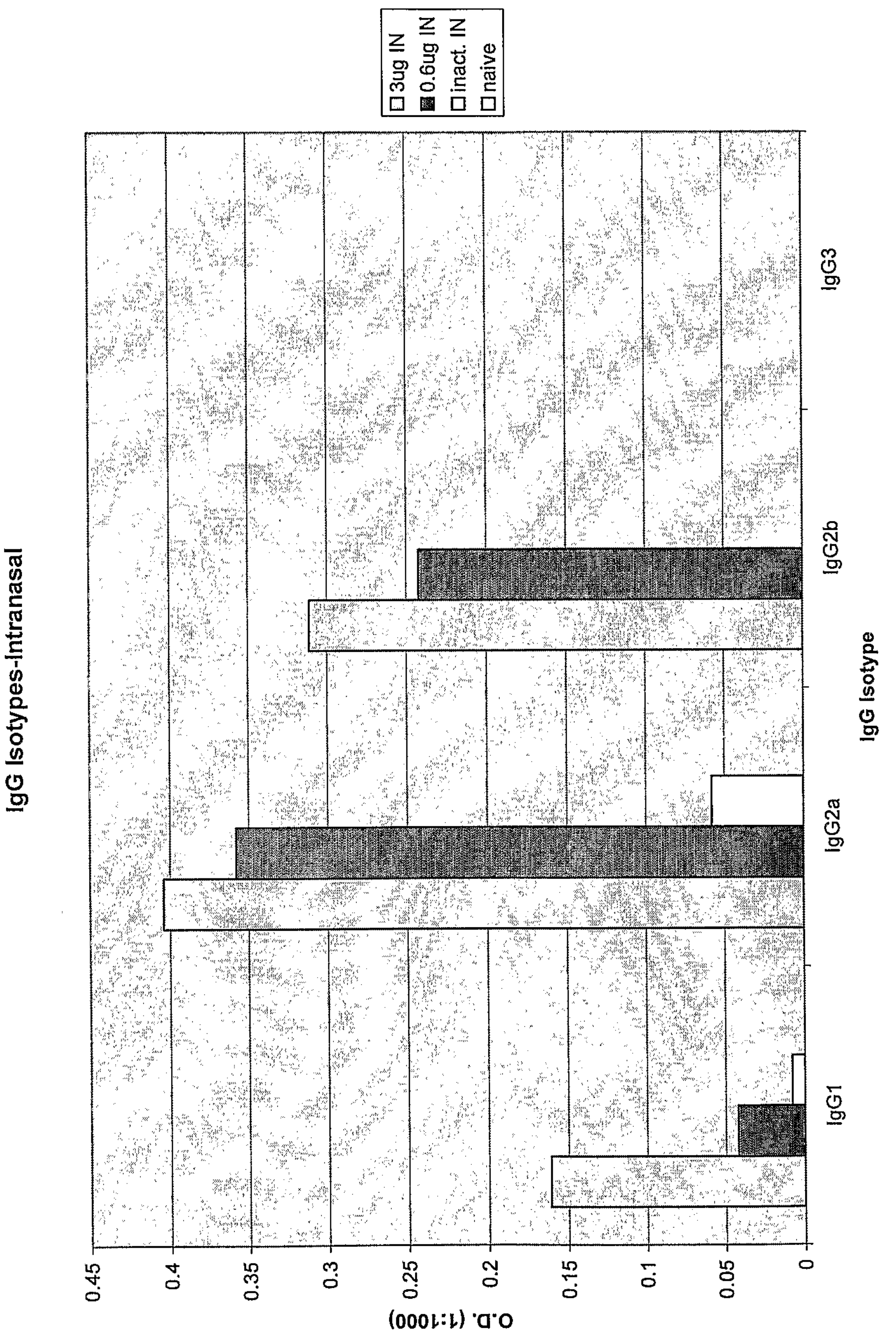

IgG Isotypes-Intranasal
O.D. (1:1000)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
IgG1
IgG2a
IgG2b
IgG3
IgG Isotype
3ug IN
0.6ug IN
inact. IN
naive

FIGURE 18 B

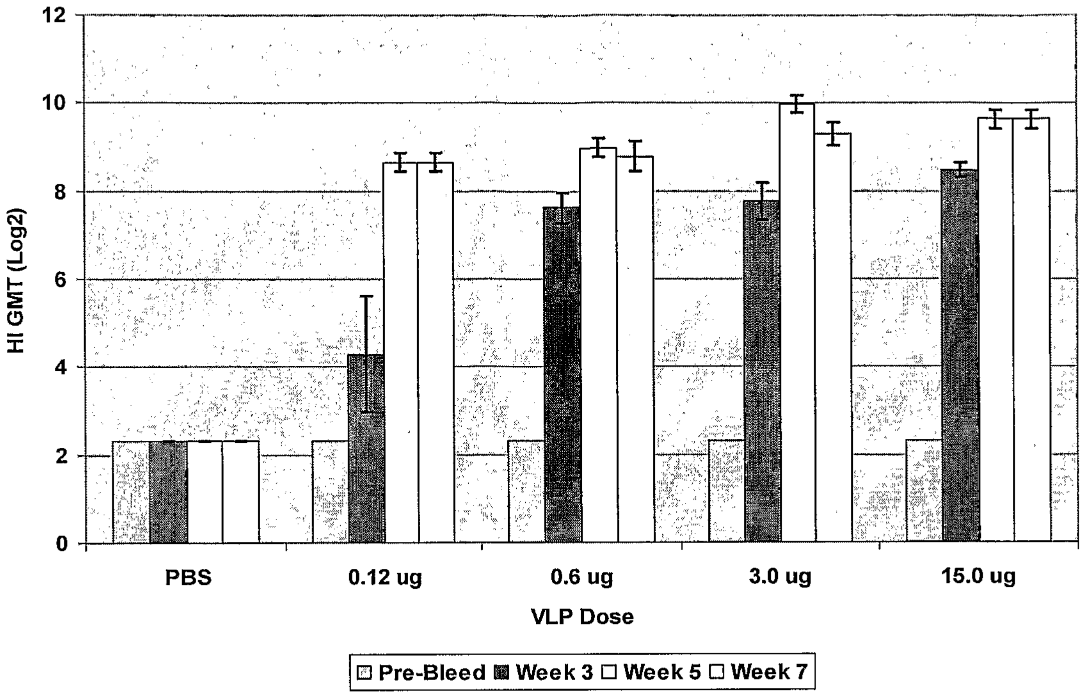

12
10
8
6
4
2
0
HI GMT (Log2)
PBS
0.12 ug
0.6 ug
3.0 ug
15.0 ug
VLP Dose
Pre-Bleed
Week 3
Week 5
Week 7

FIGURE 19

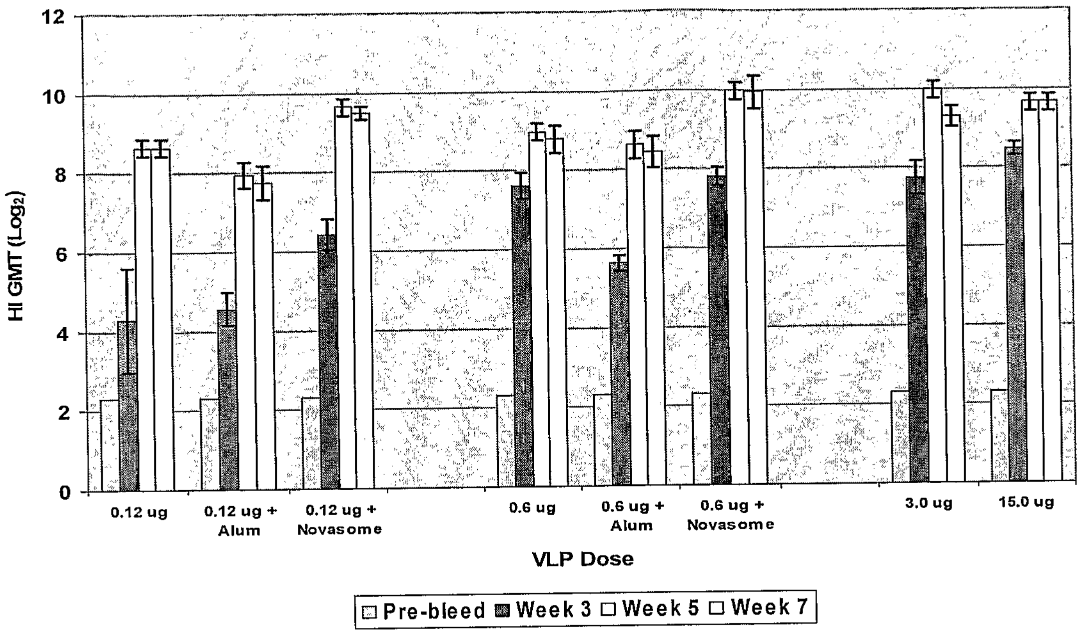

12
10
8
6
4
2
0
HI GMT (Log2)
0.12 ug
0.12 ug + Alum
0.12 ug + Novasome
0.6 ug
0.6 ug + Alum
0.6 ug + Novasome
3.0 ug
15.0 ug
VLP Dose
Pre-bleed
Week 3
Week 5
Week 7

FIGURE 20 A

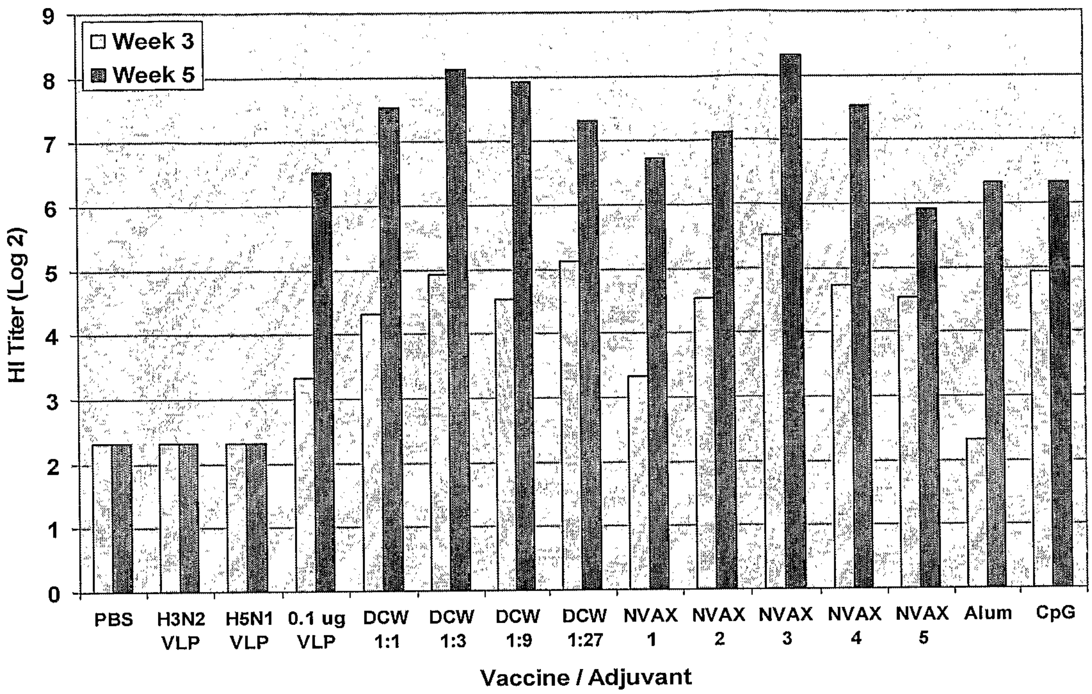

Week 3
Week 5
HI Titer (Log 2)
0
1
2
3
4
5
6
7
8
9
PBS
H3N2 VLP
H5N1 VLP
0.1 ug VLP
DCW 1:1
DCW 1:3
DCW 1:9
DCW 1:27
NVAX 1
NVAX 2
NVAX 3
NVAX 4
NVAX 5
Alum
CpG
Vaccine / Adjuvant

Figure 20 B

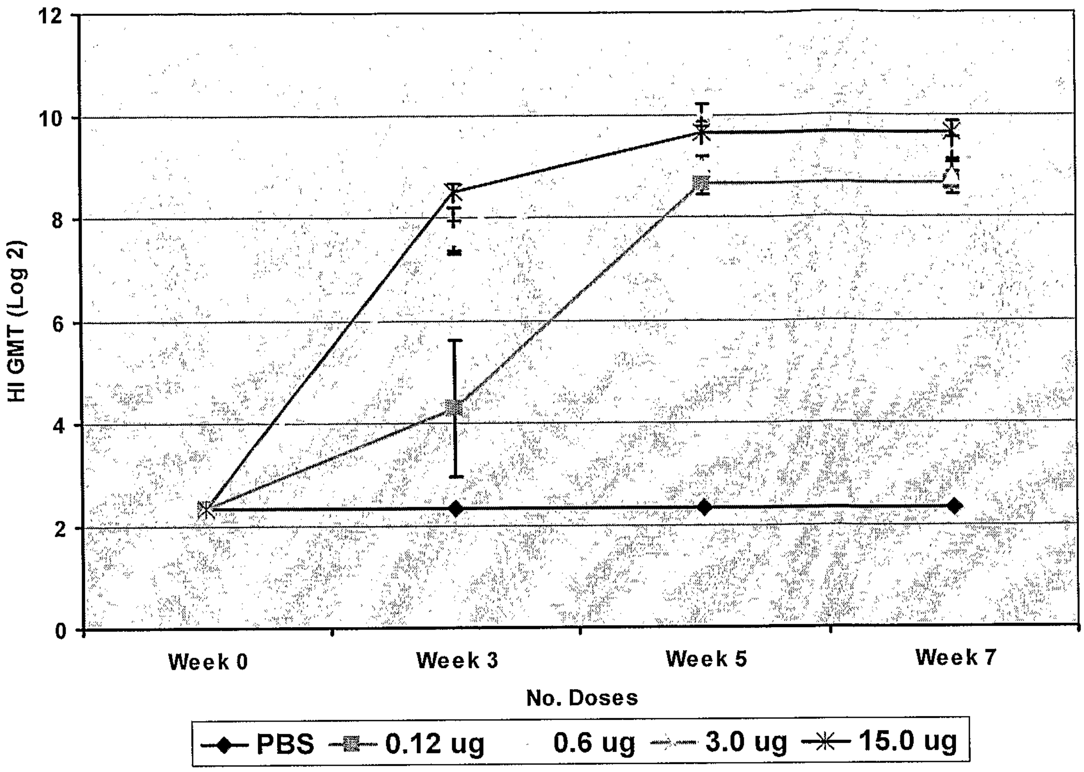

12
10
8
6
4
2
0
HI GMT (Log 2)
Week 0
Week 3
Week 5
Week 7
No. Doses
PBS
0.12 ug
0.6 ug
3.0 ug
15.0 ug

FIGURE 21

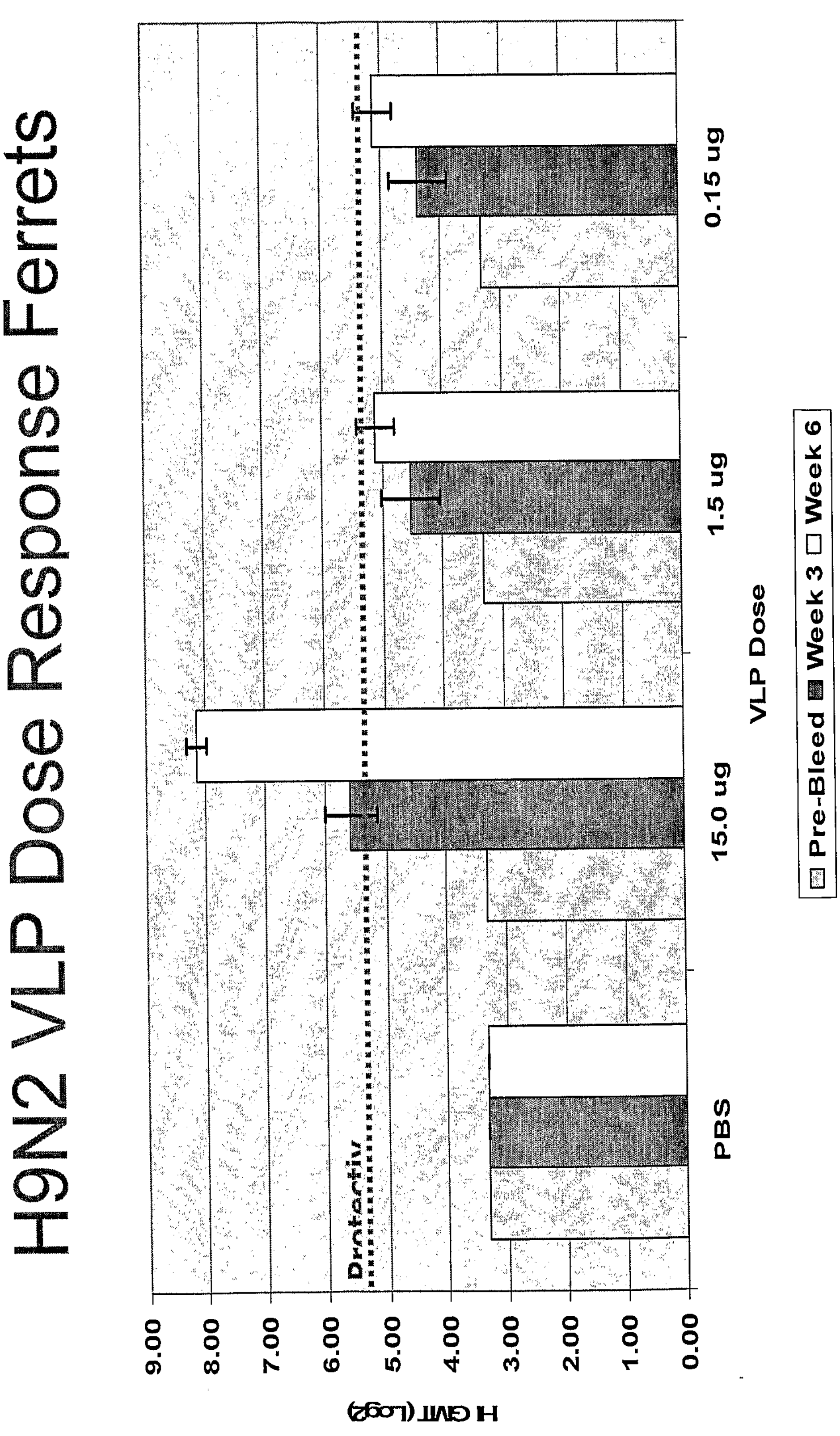

H9N2 VLP Dose Response Ferrets
H GMT (Log2)
9.00
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00
Protectiv
PBS
15.0 ug
1.5 ug
0.15 ug
VLP Dose
Pre-Bleed
Week 3
Week 6

FIGURE 22

Table X. Hemagglutinin-Inhibition Titers-Ferrets

| | H3N2 | | | | H1N1 |
|---|---|---|---|---|---|
| Vaccine | CA/04 | Fuj/02 | Well/01 | Pan/99 | NC/99 |
| Intramuscular | | | | | |
| VLP (15 ug) | 640 | 905 | 508 | 40 | 10 |
| VLP (3 ug) | 160 | 640 | 226 | 57 | 10 |
| VLP (0.6 ug) | 50 | 320 | 143 | 67 | 10 |
| VLP (0.12 ug) | 10 | 184 | 70 | 50 | 10 |
| rHA (15 ug) | 80 | 254 | 143 | 56 | 10 |
| Mock | 10 | 10 | 10 | 10 | 10 |

FIGURE 23

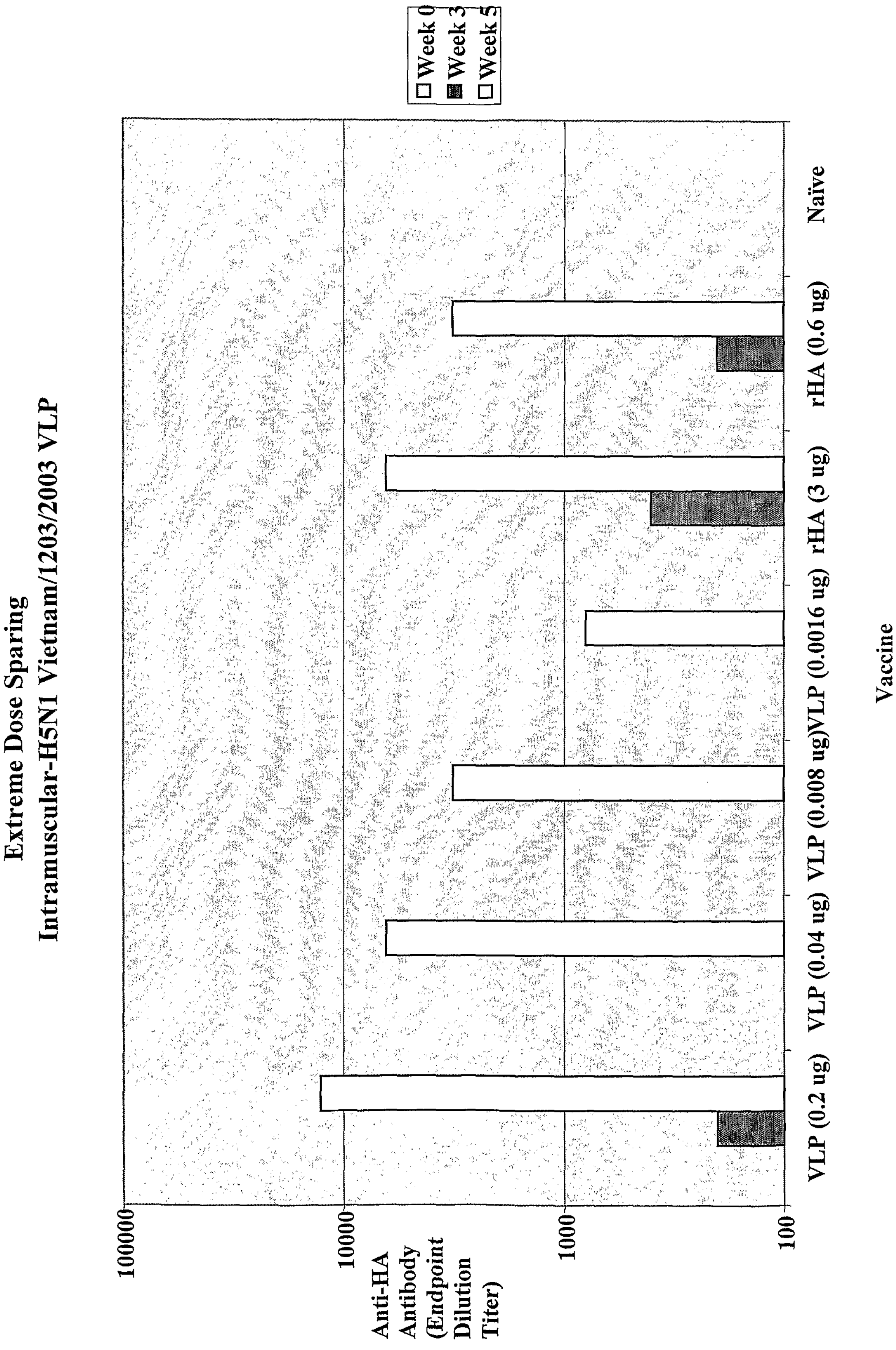

Extreme Dose Sparing
Intramuscular-H5N1 Vietnam/1203/2003 VLP
Anti-HA Antibody (Endpoint Dilution Titer)
100000
10000
1000
100
Week 0
Week 3
Week 5
VLP (0.2 ug)
VLP (0.04 ug)
VLP (0.008 ug)
VLP (0.0016 ug)
rHA (3 ug)
rHA (0.6 ug)
Naïve
Vaccine

FIGURE 24

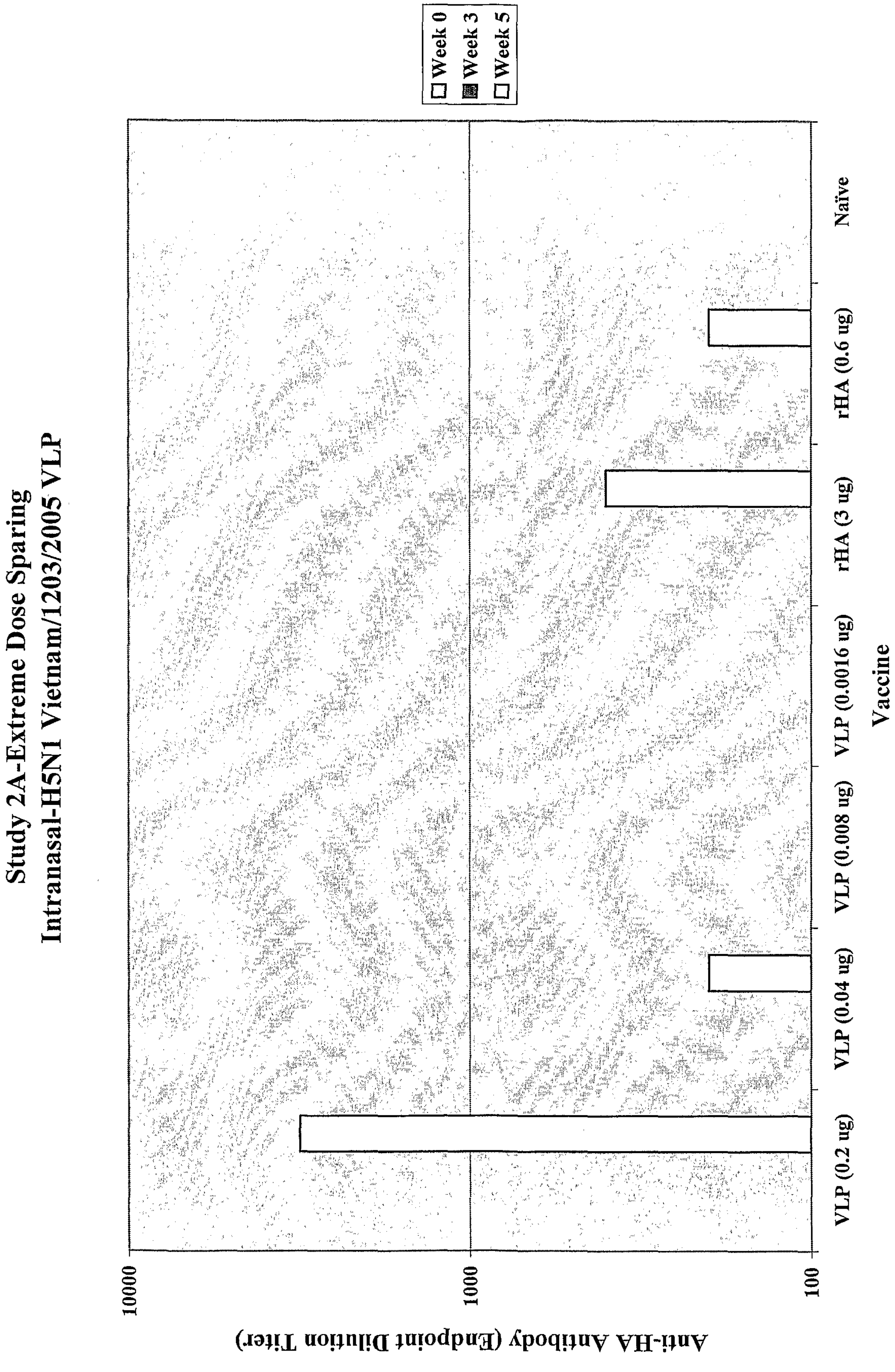

Study 2A-Extreme Dose Sparing
Intranasal-H5N1 Vietnam/1203/2005 VLP
Week 0
Week 3
Week 5
Anti-HA Antibody (Endpoint Dilution Titer)
10000
1000
100
VLP (0.2 ug)
VLP (0.04 ug)
VLP (0.008 ug)
VLP (0.0016 ug)
rHA (3 ug)
rHA (0.6 ug)
Naïve
Vaccine

FIGURE 25

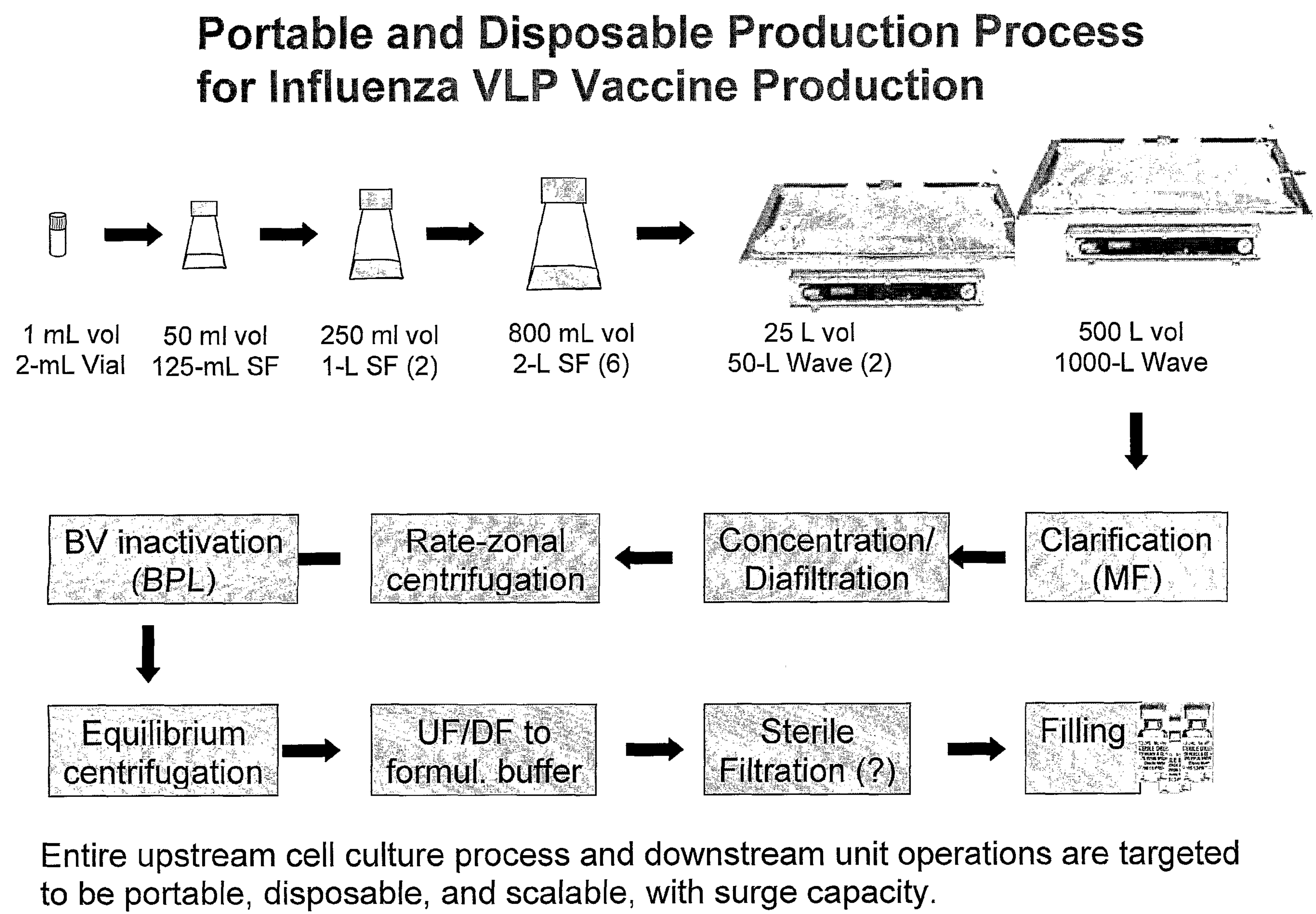



FIGURE 26

40321 v2/DC

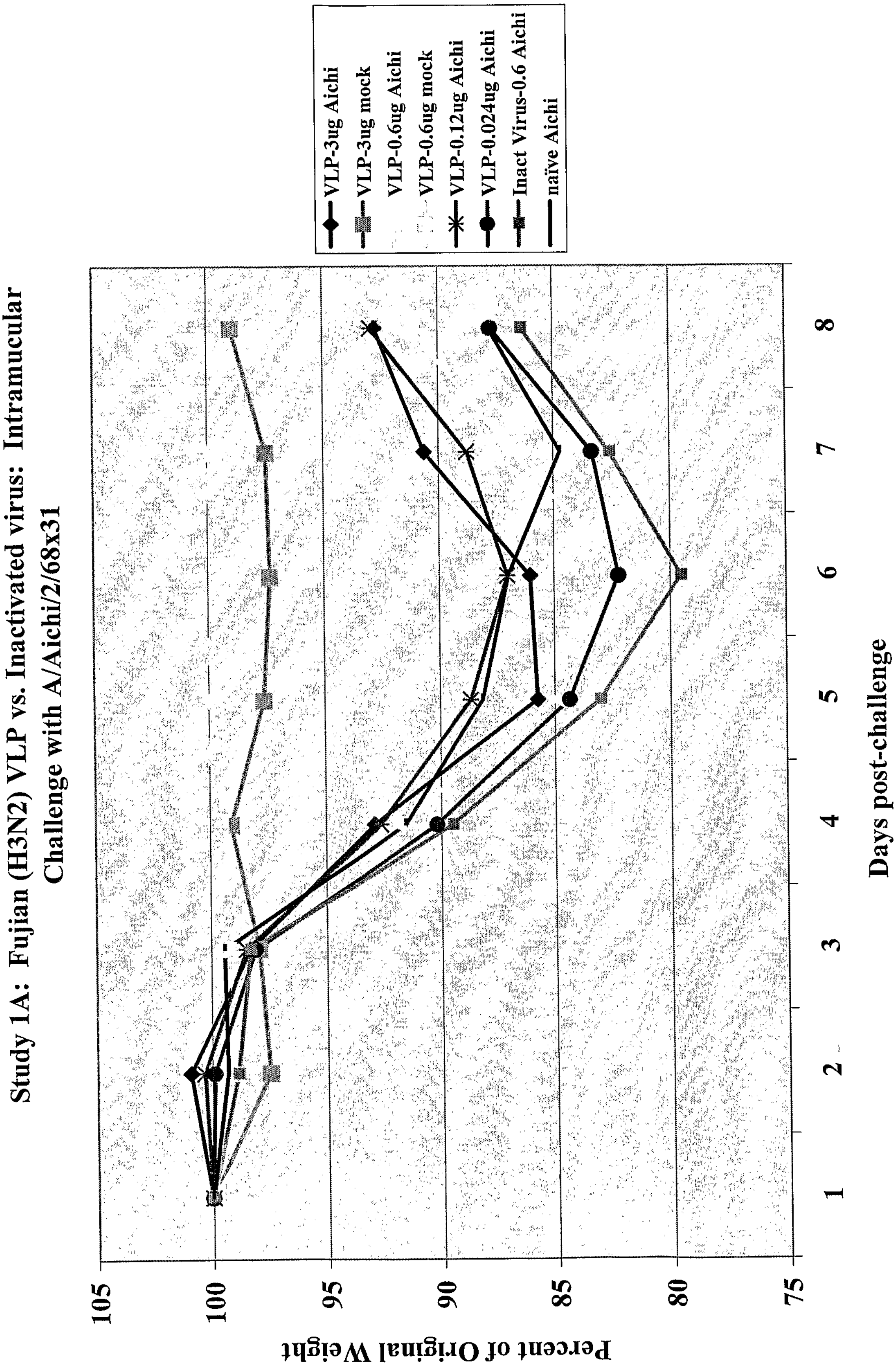

Study 1A: Fujian (H3N2) VLP vs. Inactivated virus: Intramucular Challenge with A/Aichi/2/68x31
Percent of Original Weight
105
100
95
90
85
80
75
1
2
3
4
5
6
7
8
Days post-challenge
VLP-3ug Aichi
VLP-3ug mock
VLP-0.6ug Aichi
VLP-0.6ug mock
VLP-0.12ug Aichi
VLP-0.024ug Aichi
Inact Virus-0.6 Aichi
naïve Aichi

FIGURE 27

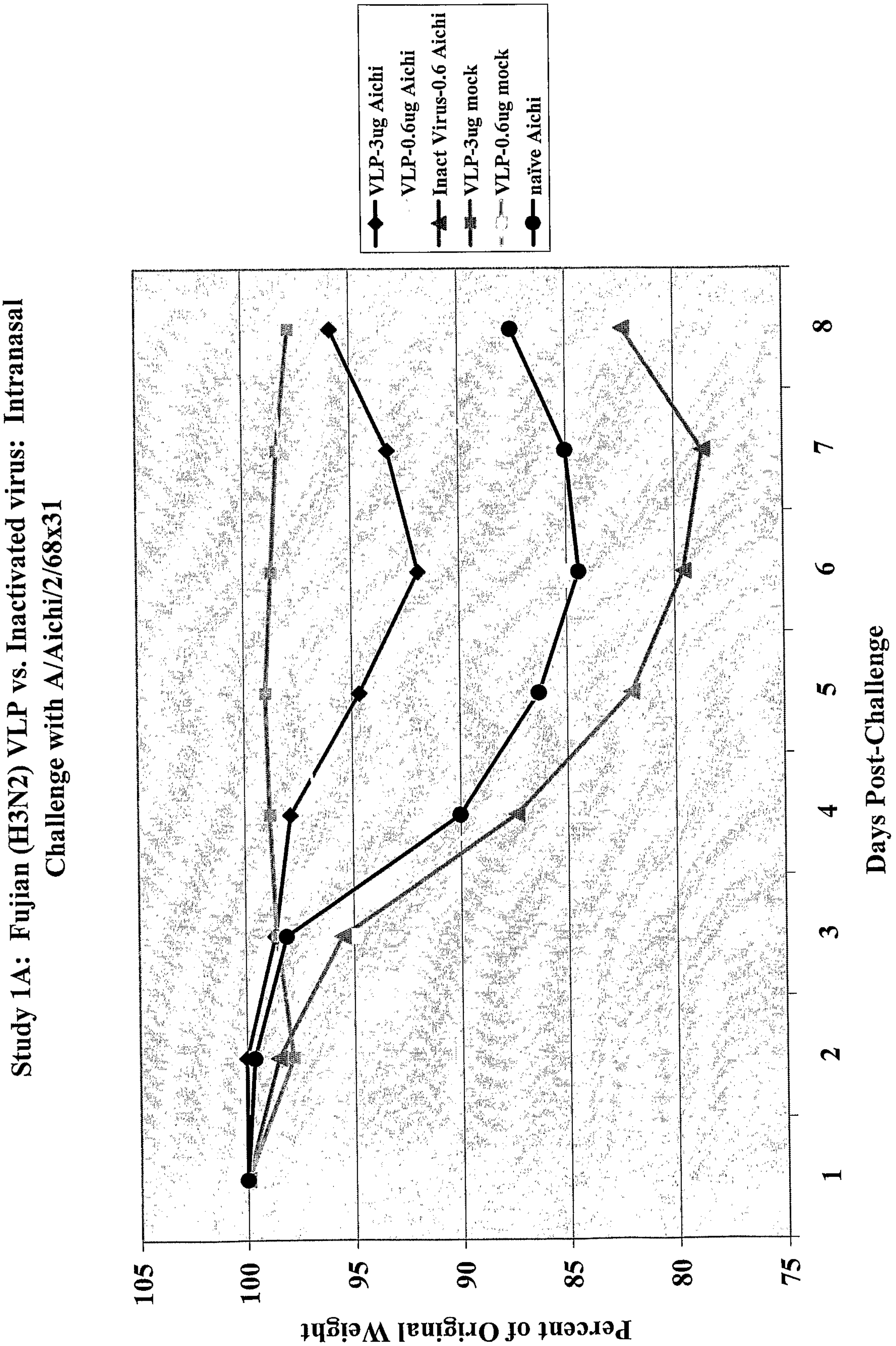

Study 1A: Fujian (H3N2) VLP vs. Inactivated virus: Intranasal Challenge with A/Aichi/2/68x31
VLP-3ug Aichi
VLP-0.6ug Aichi
Inact Virus-0.6 Aichi
VLP-3ug mock
VLP-0.6ug mock
naïve Aichi
105
100
95
90
85
80
75
Percent of Original Weight
1
2
3
4
5
6
7
8
Days Post-Challenge

FIGURE 28

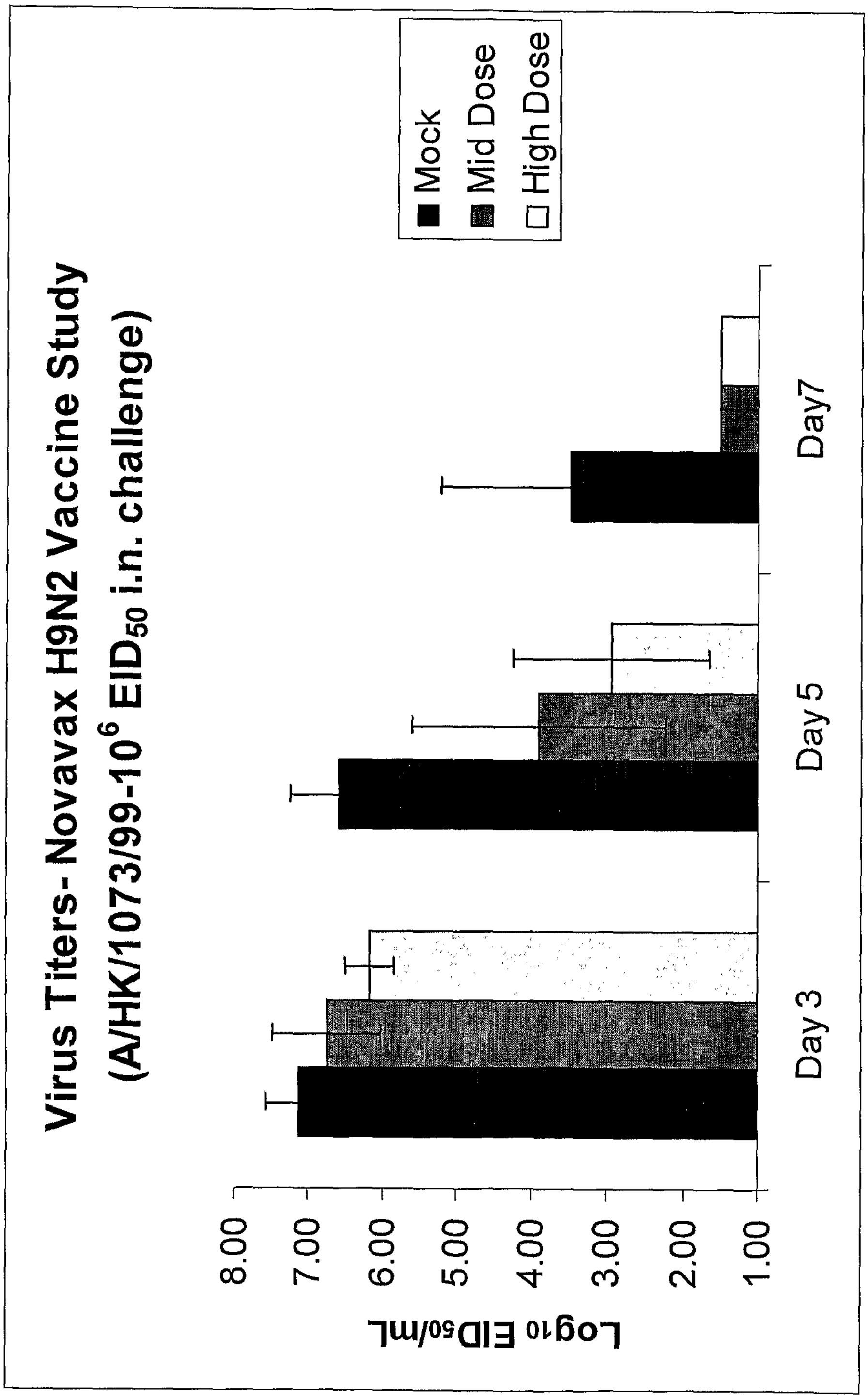
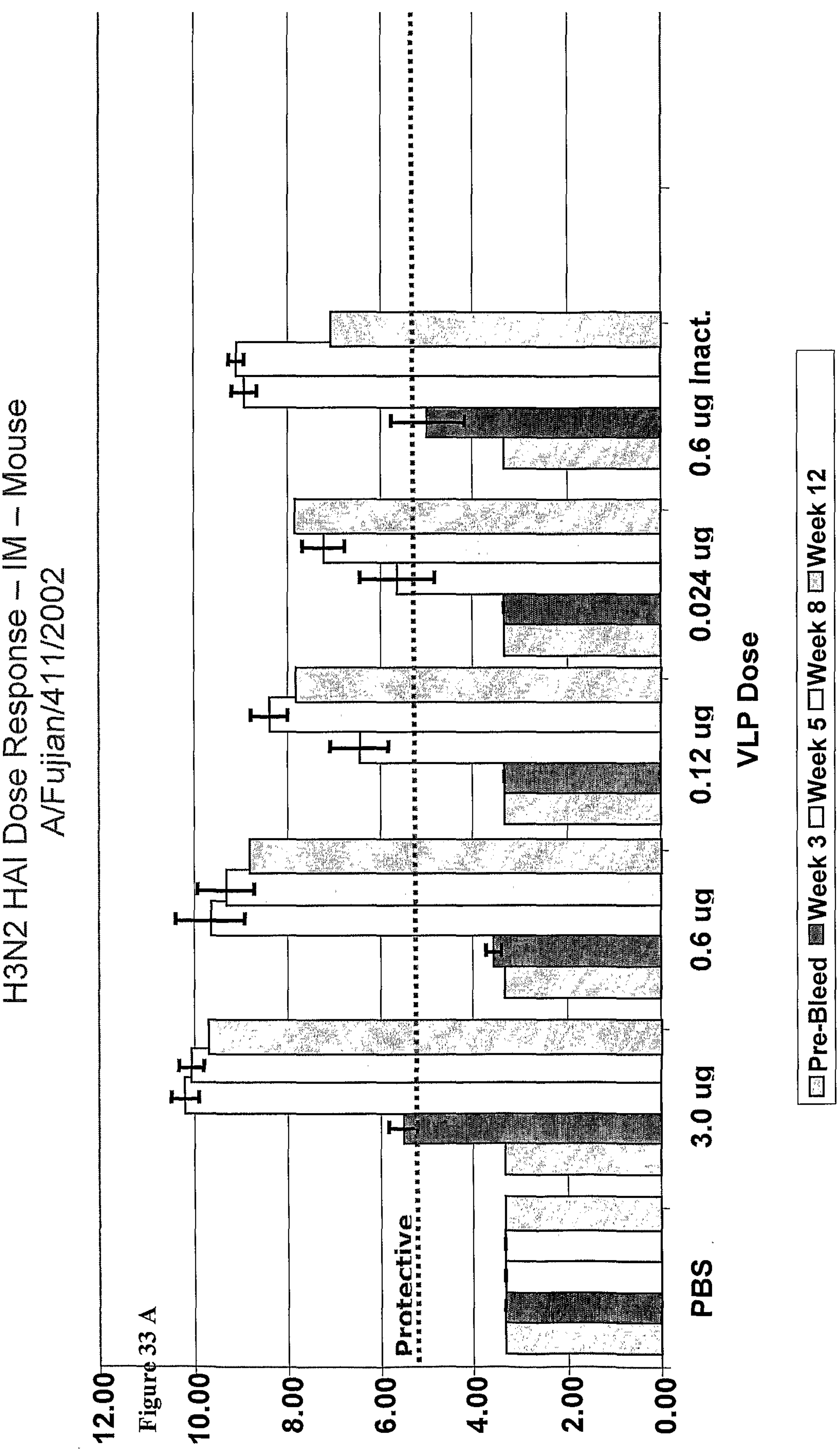

Virus Titers- Novavax H9N2 Vaccine Study
(A/HK/1073/99-10^6 EID50 i.n. challenge)
Mock
Mid Dose
High Dose
Log10 EID50/mL
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
Day 3
Day 5
Day7

FIGURE 29



H3N2 HAI Dose Response – IM – Mouse
A/Fujian/411/2002
Figure 33 A
HI GMT (Log2)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Protective
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Dose
Pre-Bleed
Week 3
Week 5
Week 8
Week 12


FIGURE 30 A

HI titer to A/Fujian/411/2002 (H3N2) after intranasal inoculation with H3H2 VLPs

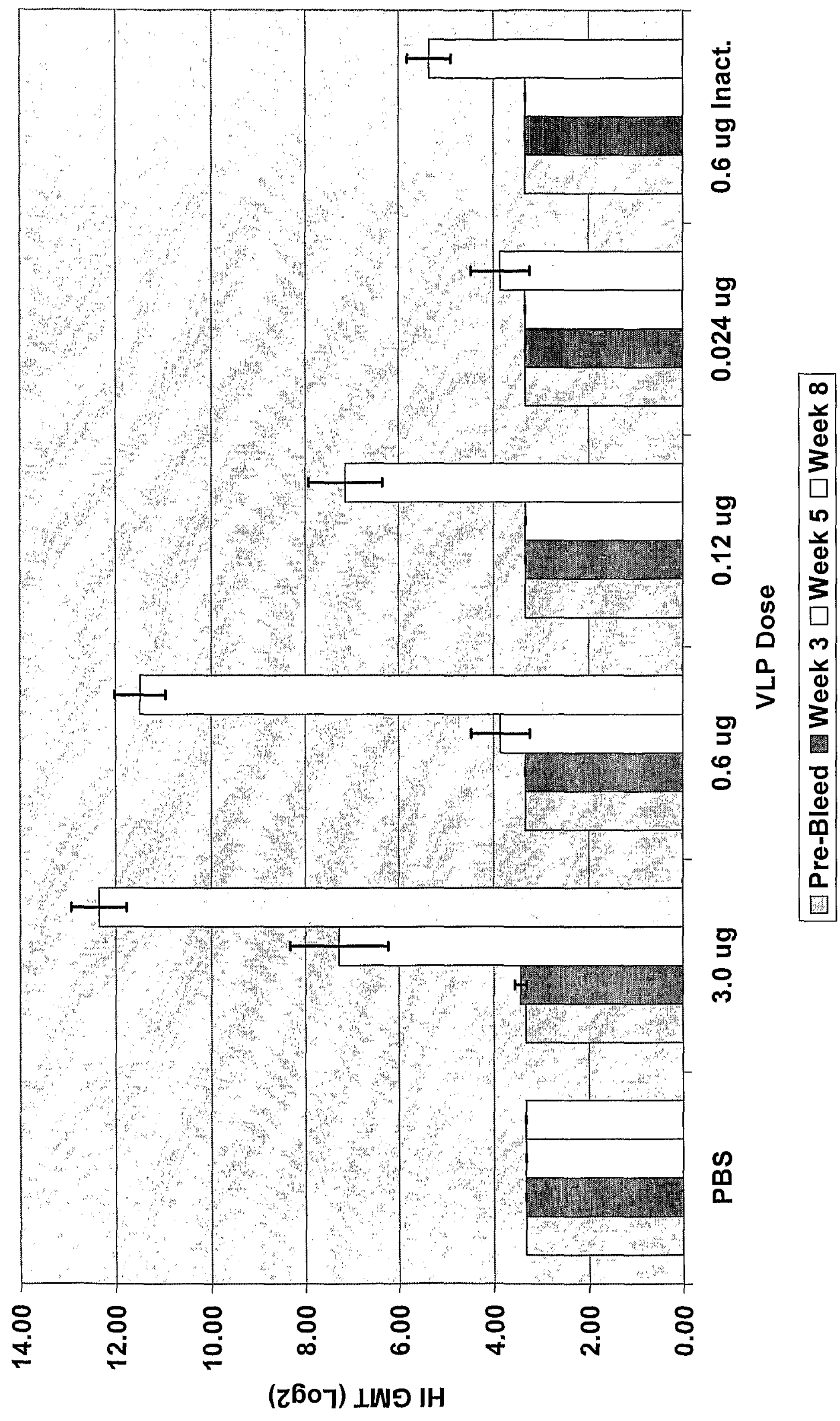



FIGURE 30 B

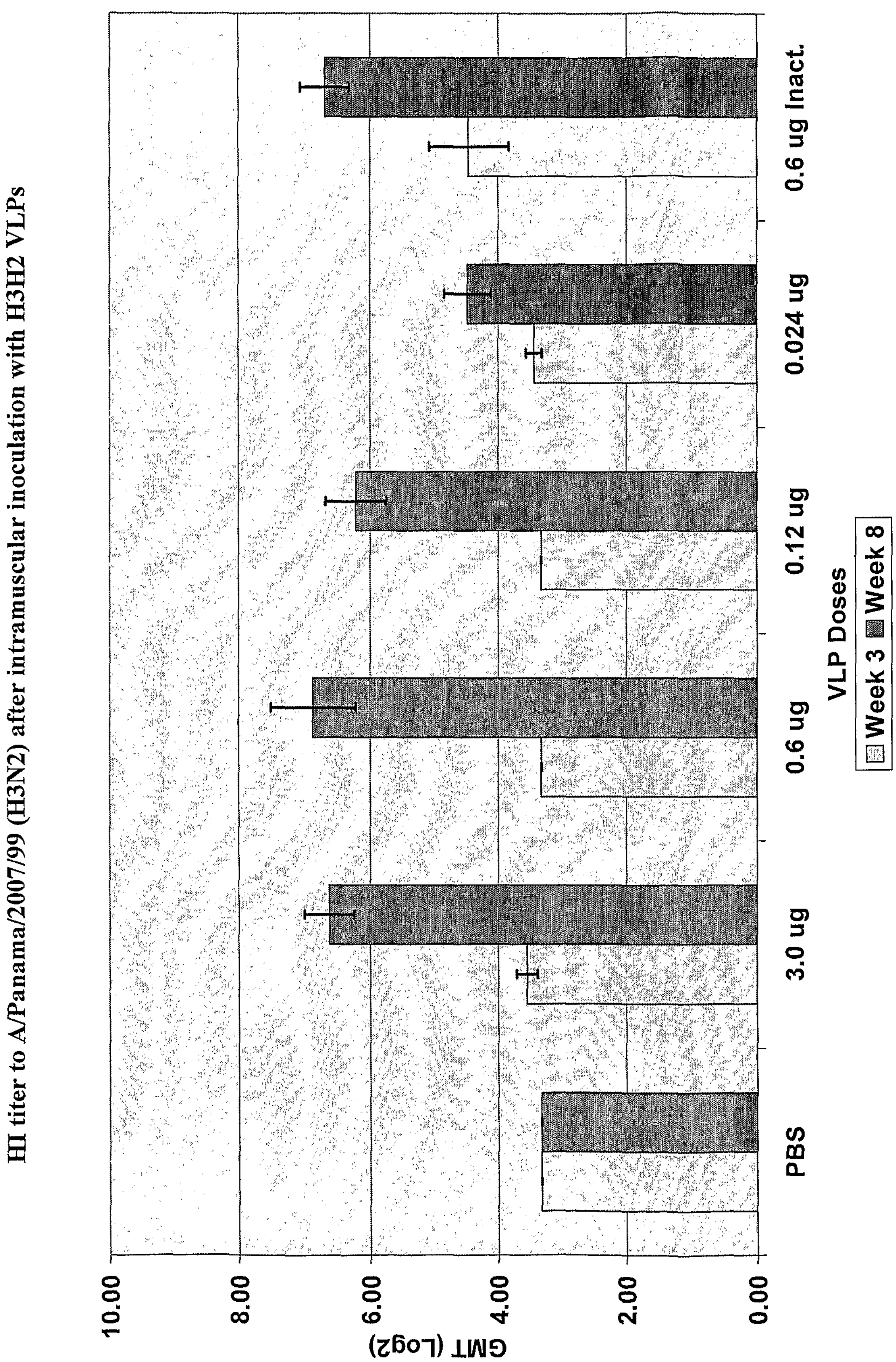

HI titer to A/Panama/2007/99 (H3N2) after intramuscular inoculation with H3H2 VLPs
GMT (Log2)
10.00
8.00
6.00
4.00
2.00
0.00
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Doses
Week 3
Week 8

FIGURE 30 C

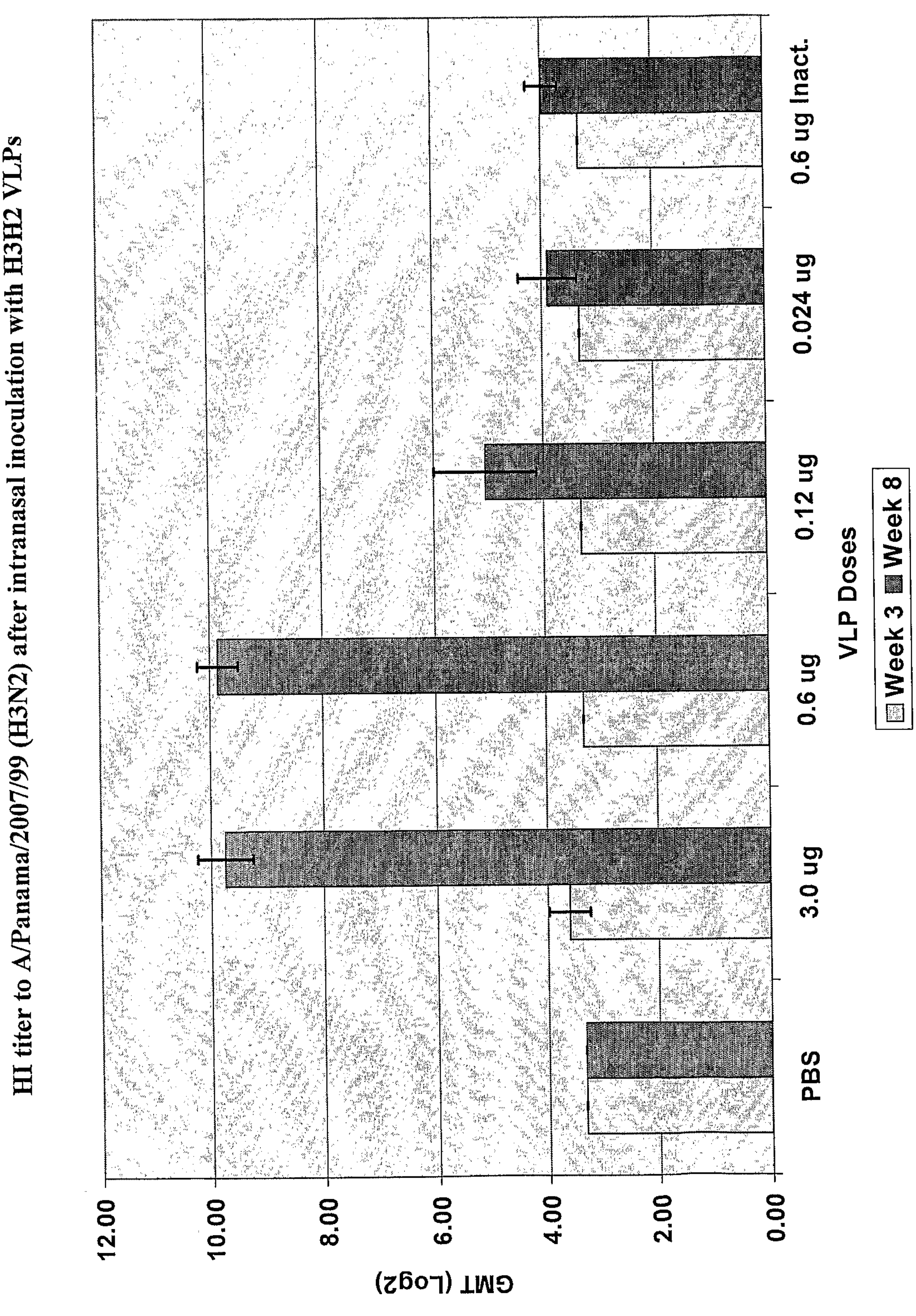

HI titer to A/Panama/2007/99 (H3N2) after intranasal inoculation with H3H2 VLPs
GMT (Log2)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Doses
Week 3
Week 8

FIGURE 30 D

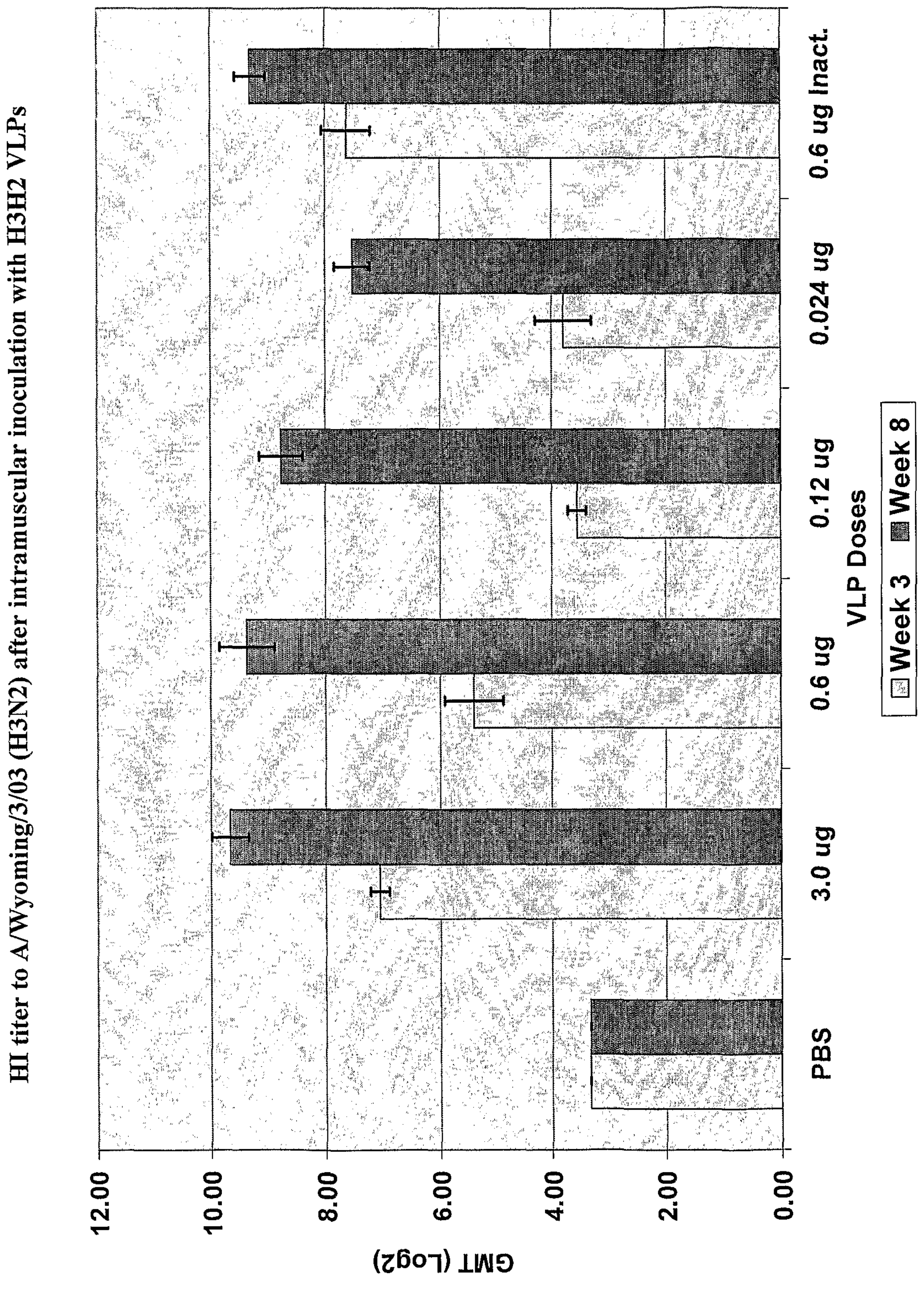

HI titer to A/Wyoming/3/03 (H3N2) after intramuscular inoculation with H3H2 VLPs
GMT (Log2)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Doses
Week 3
Week 8

FIGURE 30 E

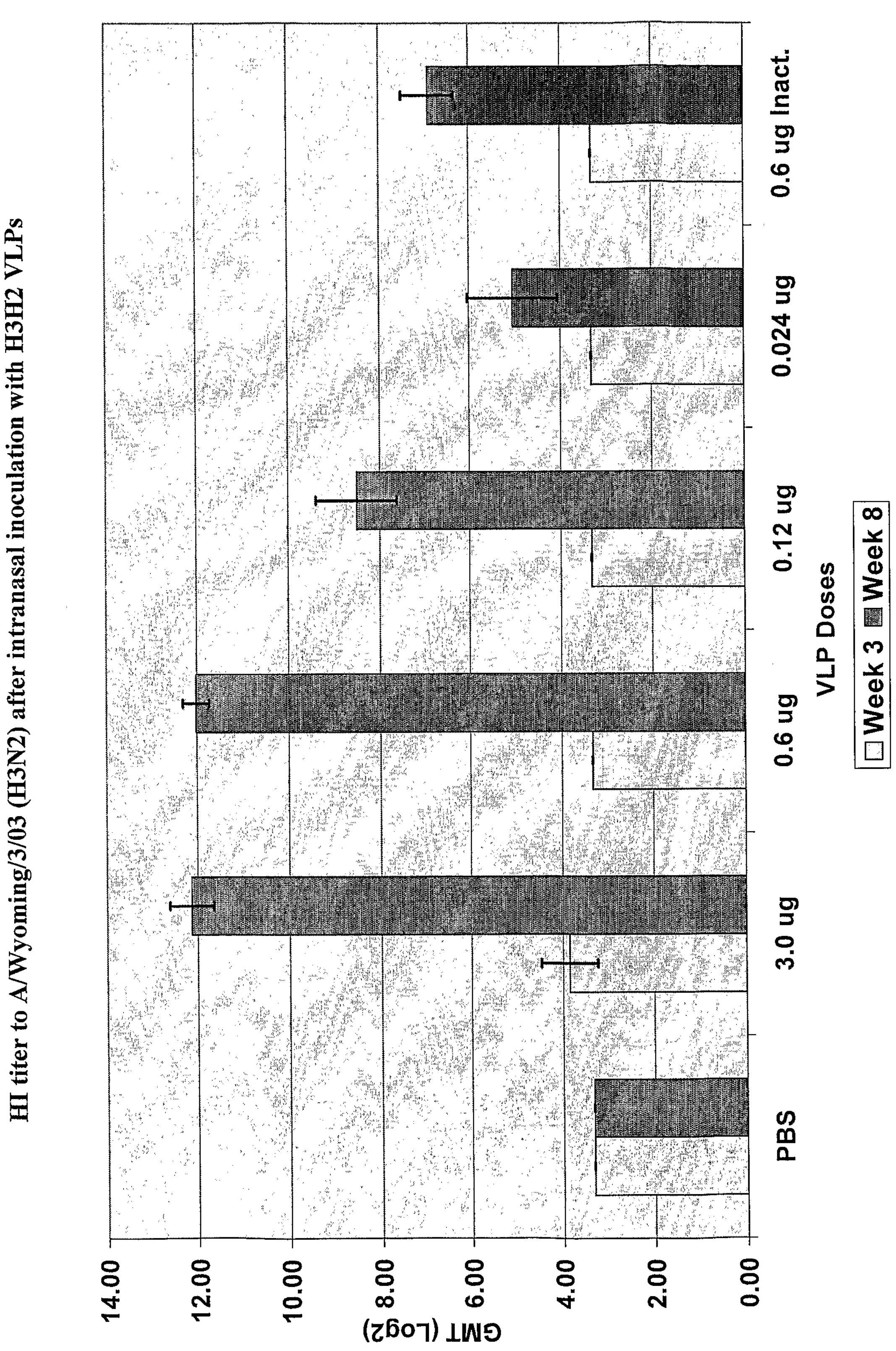

HI titer to A/Wyoming/3/03 (H3N2) after intranasal inoculation with H3H2 VLPs
GMT (Log2)
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Doses
□ Week 3 ■ Week 8

FIGURE 30 F

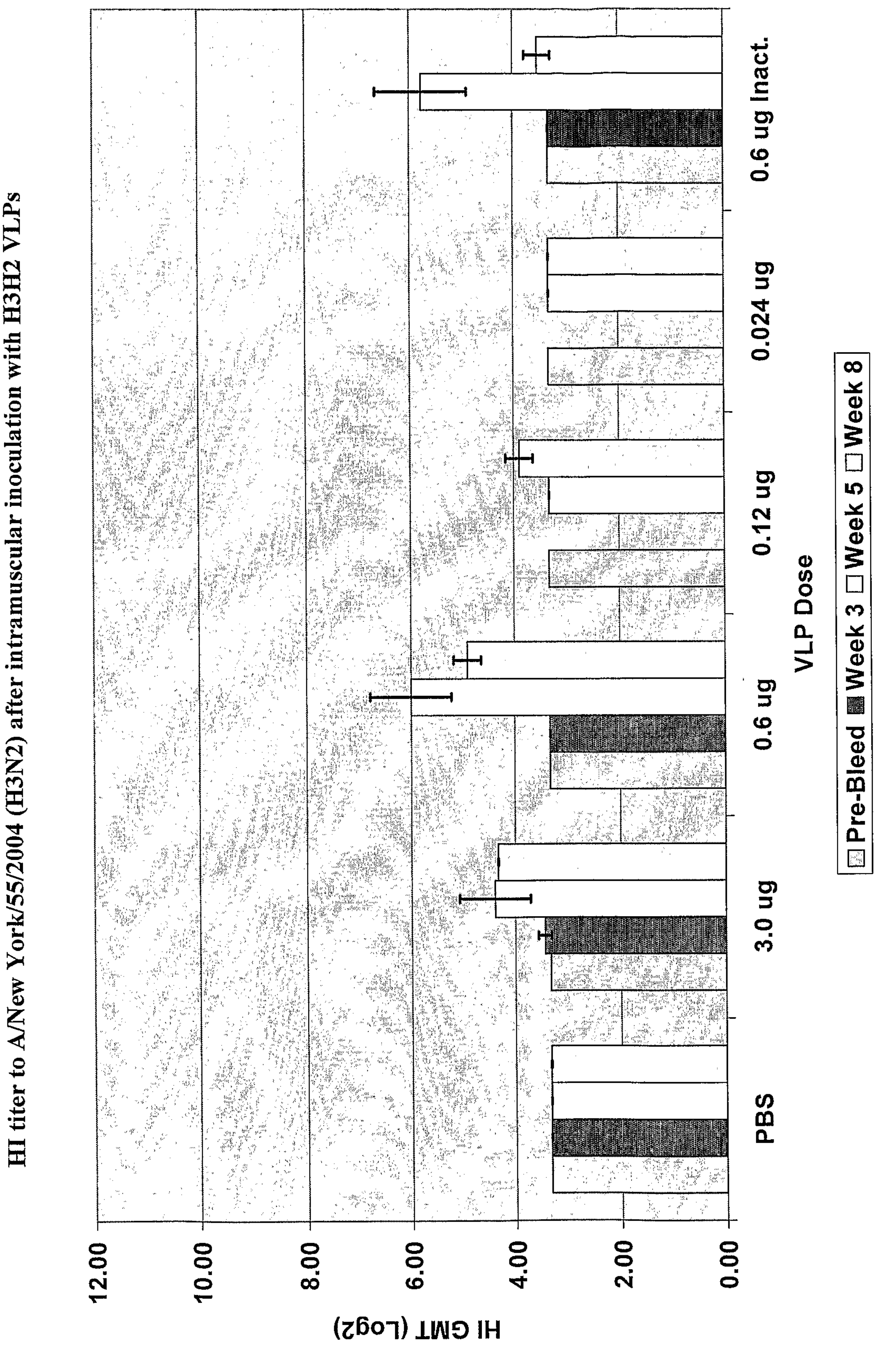

HI titer to A/New York/55/2004 (H3N2) after intramuscular inoculation with H3H2 VLPs
HI GMT (Log2)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Dose
Pre-Bleed
Week 3
Week 5
Week 8

FIGURE 30 G

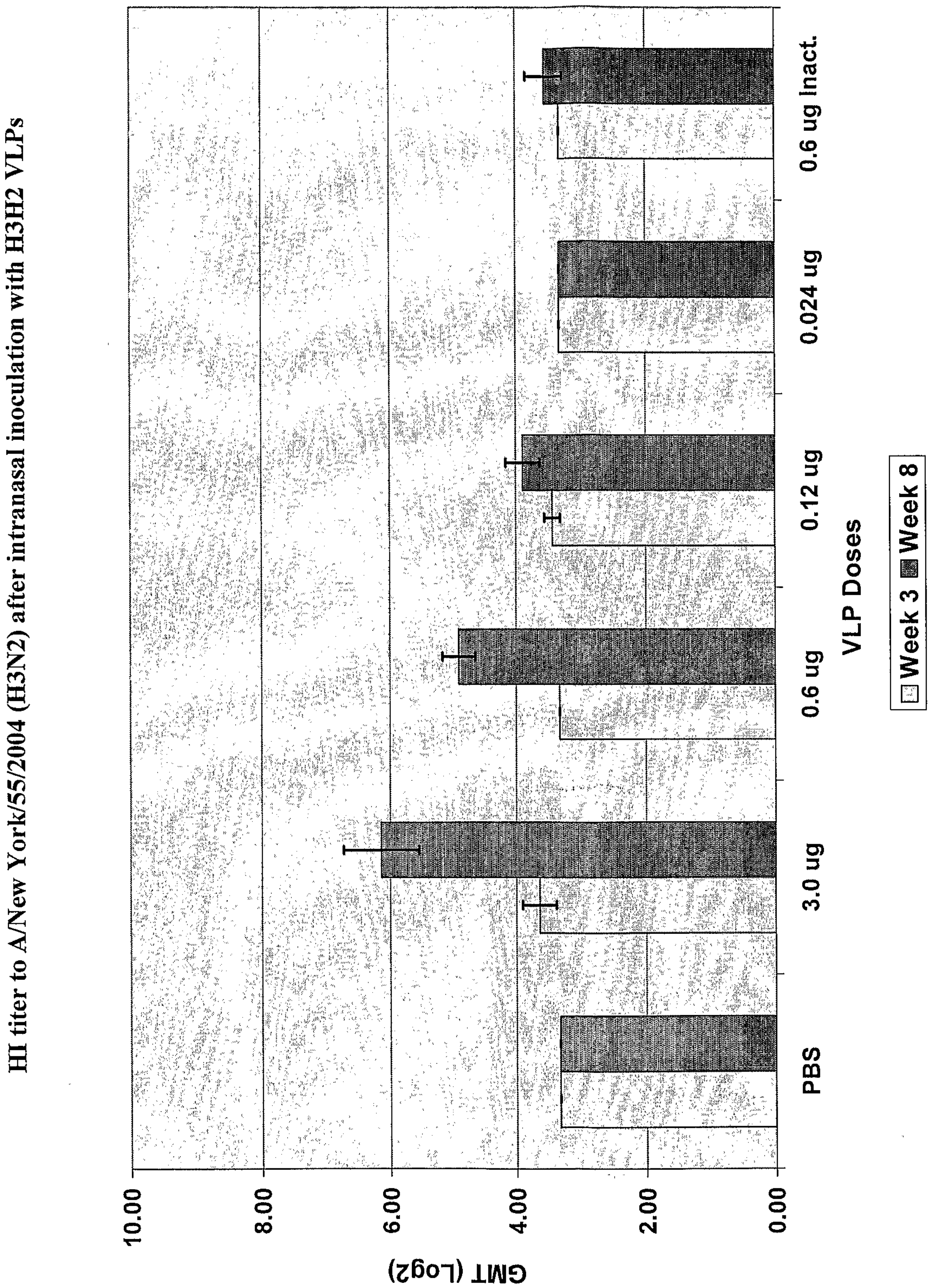

HI titer to A/New York/55/2004 (H3N2) after intranasal inoculation with H3H2 VLPs
GMT (Log2)
10.00
8.00
6.00
4.00
2.00
0.00
PBS
3.0 ug
0.6 ug
0.12 ug
0.024 ug
0.6 ug Inact.
VLP Doses
Week 3
Week 8

FIGURE 30 H

SEQUENCE LISTING

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<120> Functional Influenza Virus Like Particles (VLPs)

<130> NOVV-002/06WO

<150> US 60/727,513
<151> 2005-10-18

<150> US 60/780,847
<151> 2006-03-10

<150> US 60/800,006
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<151> 2006-07-21

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accaacccat cgaacaatca agcagtgcca tgtgaaccaa tcataataga aaggaacata 180

acagagatag tgcatttgaa taatactacc atagagaagg aaagttgtcc taaagtagca 240

gaatacaaga attggtcaaa accgcaatgt caaattacag ggttcgcccc tttctccaag 300

gacaactcaa ttaggctttc tgcaggcggg gatatttggg tgacaagaga accttatgta 360

tcgtgcggtc ttggtaaatg ttaccaattt gcacttgggc agggaaccac tttgaacaac 420

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ttgggtgttc catttcattt gggaaccaaa caagtgtgca tagcatggtc cagctcaagc 540

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gctagcatca tttatgatgg gatgcttacc gacagtattg gttcatggtc taagaacatc 660

ctcagaactc aggagtcaga atgcgtttgc atcaatggaa cttgtacagt agtaatgact 720

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gagagagggg gcccaggagt gaaagggtgg gcctttgaca atggaaatga tgtttggatg 1080

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<213> Influenza virus

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gcttcagtaa cattagcggg caattcatct ctttgcccca ttagaggatg ggctgtacac 240

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ttcatctcat gctcccacct ggaatgcaga actttcttct tgactcaggg agccttgctg 360

aatgacaagc actccaacgg gactgtcaaa gacagaagcc ctcacagaac attaatgagt 420

tgtcctgtgg gtgaggctcc ctctccatat aactcaaggt ttgagtctgt tgcttggtca 480

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gaggctgtgg ctgtattgaa atacaatggc ataataacag acactatcaa gagttggagg 600

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gtaatgactg atggaccaag tgatgggcag gcatcatata agatcttcaa aatggaaaaa 720

ggaaaagtgg tcaaatcagt cgaattggat gctcctaatt atcactatga ggaatgctcc 780

tgttatcctg atgccggcga aatcacatgt gtttgcaggg ataattggca tggctcaaat 840

aggccatggg tatctttcaa tcaaaatttg gagtatcaaa taggatatat atgcagtgga 900

gttttcggag acaatccacg ccccaatgat ggaacaggta gttgtggccc gatgtcccct 960

aacggggcat atggggtaaa agggttttca tttaaatacg gcaatggtgt ttggatcggg 1020

agaaccaaaa gcactaattc caggagcggc tttgaaatga tttgggatcc aaatgggtgg 1080

actggaacgg acagtagctt ttcagtgaaa caagatatag tagcaataac tgattggtca 1140

ggatatagcg ggagttttgt ccagcatcca gaactgacag gattagattg cataagacct 1200

tgtttctggg ttgagttaat cagagggcgg cccaaagaga gcacaatttg gactagtggg 1260

agcagcatat ctttttgtgg tgtaaatagt gacactgtga gttggtcttg gccagacggt 1320

gctgagttgc cattcaccat tgacaagtag 1350

<210> 12
<211> 759
<212> DNA
<213> Influenza virus

<400> 12
atgagtcttc taaccgaggt cgaaacgtac gttctctcta tcatcccgtc aggccccctc 60

aaagccgaga tcgcgcagaa acttgaagat gtctttgcag gaaagaacac cgatctcgag 120

gctctcatgg agtggctgaa gacaagacca atcctgtcac ctctgactaa agggattttg 180 ggatttgtat tcacgctcac cgtgcccagt gagcgaggac tgcagcgtag acgctttgtc 240

cagaatgccc taaatggaaa tggagatcca aataatatgg atagggcagt taagctatat 300

aagaagctga aaagagaaat aacattccat ggggctaaag aggtttcact cagctactca 360

accggtgcac ttgccagttg catgggtctc atatacaaca ggatgggaac ggtgactacg 420

gaagtggctt ttggcctagt gtgtgccact tgtgagcaga ttgcagattc acagcatcgg 480

tctcacaggc agatggcaac tatcaccaac ccactaatca ggcatgaaaa cagaatggtg 540

ctggccagca ctacagctaa ggctatggag cagatggcgg gatcaagtga gcaggcagcg 600

gaagccatgg aggtcgctaa tcaggctagg cagatggtgc aggcaatgag gacaattgga 660

actcatccta actctagtgc tggtctgaga gataatcttc ttgaaaattt gcaggcctac 720

cagaaacgaa tgggagtgca gatgcagcga ttcaagtga 759

<210> 13
<211> 30
<212> DNA
<213> Influenza virus

<400> 13
aggatccatg aagactatca ttgctttgag 30

<210> 14
<211> 32
<212> DNA
<213> Influenza virus

<400> 14
aggtacctca aatgcaaatg ttgcacctaa tg 32

<210> 15
<211> 72
<212> DNA
<213> Influenza virus

<400> 15
ggggacaagt ttgtacaaaa aagcaggctt agaaggagat agaaccatga atccaaatca 60

aaagataata ac 72

<210> 16
<211> 57
<212> DNA
<213> Influenza virus

<400> 16
ggggaccact ttgtacaaga aagctgggtc ctatataggc atgagattga tgtccgc 57

<210> 17

<211> 38
<212> DNA
<213> Influenza virus

<400> 17
aaagaattca tgagtcttct aaccgaggtc gaaacgta 38

<210> 18
<211> 38
<212> DNA
<213> Influenza virus

<400> 18
aaattcgaat tactccagct ctatgctgac aaaatgac 38

<210> 19
<211> 57
<212> DNA
<213> Influenza virus

<400> 19
agaatcatga gtcttctaac cgaggtcgaa acgcctatca gaaacgaatg gggtgc 57

<210> 20
<211> 38
<212> DNA
<213> Influenza virus

<400> 20
aaattcgaat tactccagct ctatgctgac aaaatgac 38

<210> 21
<211> 30
<212> DNA
<213> Influenza virus

<400> 21
agaattcatg gcgtcccaag gcaccaaacg 30

<210> 22
<211> 50
<212> DNA
<213> Influenza virus

<400> 22
agcggccgct taattgtcgt actcctctgc attgtctccg aagaaataag 50

<210> 23
<211> 35
<212> DNA
<213> Influenza virus

<400> 23
agaattcatg aaggcaataa ttgtactact catgg 35

<210> 24
<211> 47
<212> DNA
<213> Influenza virus

<400> 24
agcggccgct tatagacaga tggagcaaga aacattgtct ctggaga 47

<210> 25
<211> 31
<212> DNA
<213> Influenza virus

<400> 25
agaattcatg ctaccttcaa ctatacaaac g 31

<210> 26
<211> 40
<212> DNA
<213> Influenza virus

<400> 26
agcggccgct tacagagcca tatcaacacc tgtgacagtg 40

<210> 27
<211> 568
<212> PRT
<213> Unknown

<220>
<223> Vac2-hac-opt

<400> 27

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
35 40 45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
50 55 60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65 70 75 80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
85 90 95

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Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser Phe Asn
            100                 105                 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
        115                 120                 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
    130                 135                 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser Phe Phe
145                 150                 155                 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro Thr Ile
                165                 170                 175

Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
            180                 185                 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
        195                 200                 205

Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn Gln Arg
    210                 215                 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225                 230                 235                 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
                245                 250                 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
            260                 265                 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
        275                 280                 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
    290                 295                 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305                 310                 315                 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
                325                 330                 335
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Pro Gln Arg Glu Ser Arg Arg Lys Lys Arg Gly Leu Phe Gly Ala Ile
340 345 350

Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr
355 360 365

Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys
370 375 380

Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser
385 390 395 400

Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe
405 410 415

Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp
420 425 430

Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met
435 440 445

Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu
450 455 460

Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly
465 470 475 480

Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu
485 490 495

Ser Ile Arg Asn Gly Thr Tyr Asn Tyr Pro Gln Tyr Ser Glu Glu Ala
500 505 510

Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly
515 520 525

Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala
530 535 540

Leu Ala Ile Met Met Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly
545 550 555 560

Ser Leu Gln Cys Arg Ile Cys Ile
565

<210> 28

<211> 572
<212> PRT
<213> Unknown

<220>
<223> Vac2-hac-spc-opt

<400> 28

Met Pro Leu Tyr Lys Leu Leu Asn Val Leu Trp Leu Val Ala Val Ser
1 5 10 15

Asn Ala Ile Pro Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser
20 25 30

Thr Glu Gln Val Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His
35 40 45

Ala Gln Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu
50 55 60

Asp Gly Val Lys Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp
65 70 75 80

Leu Leu Gly Asn Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp
85 90 95

Ser Tyr Ile Val Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro
100 105 110

Gly Ser Phe Asn Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile
115 120 125

Asn His Phe Glu Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp
130 135 140

His Glu Ala Ser Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser
145 150 155 160

Pro Ser Phe Phe Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr
165 170 175

Tyr Pro Thr Ile Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu
180 185 190

Leu Val Leu Trp Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr
195 200 205

Arg Leu Tyr Gln Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr
210 215 220

Leu Asn Gln Arg Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn
225 230 235 240

Gly Gln Ser Gly Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn
245 250 255

Asp Ala Ile Asn Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr
260 265 270

Ala Tyr Lys Ile Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu
275 280 285

Leu Glu Tyr Gly Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala
290 295 300

Ile Asn Ser Ser Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly
305 310 315 320

Glu Cys Pro Lys Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly
325 330 335

Leu Arg Asn Ser Pro Gln Arg Glu Ser Arg Arg Lys Lys Arg Gly Leu
340 345 350

Phe Gly Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val
355 360 365

Asp Gly Trp Tyr Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr
370 375 380

Ala Ala Asp Lys Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn
385 390 395 400

Lys Val Asn Ser Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val
405 410 415

Gly Arg Glu Phe Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys
420 425 430

Lys Met Glu Asp Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu
435 440 445

Leu Val Leu Met Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn

450 455 460

Val Lys Asn Leu Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala
465 470 475 480

Lys Glu Leu Gly Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn
485 490 495

Glu Cys Met Glu Ser Ile Arg Asn Gly Thr Tyr Asn Tyr Pro Gln Tyr
500 505 510

Ser Glu Glu Ala Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu
515 520 525

Glu Ser Ile Gly Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala
530 535 540

Ser Ser Leu Ala Leu Ala Ile Met Met Ala Gly Leu Ser Leu Trp Met
545 550 555 560

Cys Ser Asn Gly Ser Leu Gln Cys Arg Ile Cys Ile
565 570

<210> 29
<211> 570
<212> PRT
<213> Unknown

<220>
<223> Vac2-hac-sph9-opt

<400> 29

Met Glu Thr Ile Ser Leu Ile Thr Ile Leu Leu Val Val Thr Ala Ser
1 5 10 15

Asn Ala Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu
20 25 30

Gln Val Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln
35 40 45

Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly
50 55 60

Val Lys Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu
65 70 75 80

Gly Asn Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr
85 90 95

Ile Val Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser
100 105 110

Phe Asn Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His
115 120 125

Phe Glu Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu
130 135 140

Ala Ser Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser
145 150 155 160

Phe Phe Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro
165 170 175

Thr Ile Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val
180 185 190

Leu Trp Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu
195 200 205

Tyr Gln Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn
210 215 220

Gln Arg Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln
225 230 235 240

Ser Gly Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala
245 250 255

Ile Asn Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr
260 265 270

Lys Ile Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu
275 280 285

Tyr Gly Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn
290 295 300

Ser Ser Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys
305 310 315 320

Pro Lys Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg

325 330 335

Asn Ser Pro Gln Arg Glu Ser Arg Arg Lys Lys Arg Gly Leu Phe Gly
340 345 350

Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly
355 360 365

Trp Tyr Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala
370 375 380

Asp Lys Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn Lys Val
385 390 395 400

Asn Ser Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val Gly Arg
405 410 415

Glu Phe Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys Lys Met
420 425 430

Glu Asp Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu Leu Val
435 440 445

Leu Met Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn Val Lys
450 455 460

Asn Leu Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu
465 470 475 480

Leu Gly Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn Glu Cys
485 490 495

Met Glu Ser Ile Arg Asn Gly Thr Tyr Asn Tyr Pro Gln Tyr Ser Glu
500 505 510

Glu Ala Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu Glu Ser
515 520 525

Ile Gly Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser
530 535 540

Leu Ala Leu Ala Ile Met Met Ala Gly Leu Ser Leu Trp Met Cys Ser
545 550 555 560

Asn Gly Ser Leu Gln Cys Arg Ile Cys Ile
565 570

<210> 30
<211> 564
<212> PRT
<213> Unknown

<220>
<223> Vac2-hac-cs-opt

<400> 30

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
35 40 45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
50 55 60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65 70 75 80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
85 90 95

Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser Phe Asn
100 105 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
115 120 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
130 135 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser Phe Phe
145 150 155 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro Thr Ile
165 170 175

Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
180 185 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln

195 200 205

Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn Gln Arg
210 215 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225 230 235 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
245 250 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
260 265 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
275 280 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
290 295 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305 310 315 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
325 330 335

Pro Gln Arg Glu Ser Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile
340 345 350

Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr Gly Tyr His His
355 360 365

Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys Glu Ser Thr Gln
370 375 380

Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser Ile Ile Asp Lys
385 390 395 400

Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe Asn Asn Leu Glu
405 410 415

Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp Gly Phe Leu Asp
420 425 430

Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met Glu Asn Glu Arg
435 440 445

Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu Tyr Asp Lys Val
450 455 460

Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly Asn Gly Cys Phe
465 470 475 480

Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu Ser Ile Arg Asn
485 490 495

Gly Thr Tyr Asn Tyr Pro Gln Tyr Ser Glu Glu Ala Arg Leu Lys Arg
500 505 510

Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly Thr Tyr Gln Ile
515 520 525

Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala Leu Ala Ile Met
530 535 540

Met Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly Ser Leu Gln Cys
545 550 555 560

Arg Ile Cys Ile

<210> 31
<211> 449
<212> PRT
<213> Unknown

<220>
<223> Vac2-naj-opt

<400> 31

Met Asn Pro Asn Gln Lys Ile Ile Thr Ile Gly Ser Ile Cys Met Val
1 5 10 15

Ile Gly Ile Val Ser Leu Met Leu Gln Ile Gly Asn Met Ile Ser Ile
20 25 30

Trp Val Ser His Ser Ile Gln Thr Gly Asn Gln His Gln Ala Glu Ser
35 40 45

Ile Ser Asn Thr Asn Pro Leu Thr Glu Lys Ala Val Ala Ser Val Thr
50 55 60

Leu Ala Gly Asn Ser Ser Leu Cys Pro Ile Arg Gly Trp Ala Val His

65 70 75 80

Ser Lys Asp Asn Asn Ile Arg Ile Gly Ser Lys Gly Asp Val Phe Val
85 90 95

Ile Arg Glu Pro Phe Ile Ser Cys Ser His Leu Glu Cys Arg Thr Phe
100 105 110

Phe Leu Thr Gln Gly Ala Leu Leu Asn Asp Lys His Ser Asn Gly Thr
115 120 125

Val Lys Asp Arg Ser Pro His Arg Thr Leu Met Ser Cys Pro Val Gly
130 135 140

Glu Ala Pro Ser Pro Tyr Asn Ser Arg Phe Glu Ser Val Ala Trp Ser
145 150 155 160

Ala Ser Ala Cys His Asp Gly Thr Ser Trp Leu Thr Ile Gly Ile Ser
165 170 175

Gly Pro Asp Asn Glu Ala Val Ala Val Leu Lys Tyr Asn Gly Ile Ile
180 185 190

Thr Asp Thr Ile Lys Ser Trp Arg Asn Asn Ile Leu Arg Thr Gln Glu
195 200 205

Ser Glu Cys Ala Cys Val Asn Gly Ser Cys Phe Thr Val Met Thr Asp
210 215 220

Gly Pro Ser Asp Gly Gln Ala Ser Tyr Lys Ile Phe Lys Met Glu Lys
225 230 235 240

Gly Lys Val Val Lys Ser Val Glu Leu Asp Ala Pro Asn Tyr His Tyr
245 250 255

Glu Glu Cys Ser Cys Tyr Pro Asp Ala Gly Glu Ile Thr Cys Val Cys
260 265 270

Arg Asp Asn Trp His Gly Ser Asn Arg Pro Trp Val Ser Phe Asn Gln
275 280 285

Asn Leu Glu Tyr Gln Ile Gly Tyr Ile Cys Ser Gly Val Phe Gly Asp
290 295 300

Asn Pro Arg Pro Asn Asp Gly Thr Gly Ser Cys Gly Pro Met Ser Pro
305 310 315 320

Asn Gly Ala Tyr Gly Val Lys Gly Phe Ser Phe Lys Tyr Gly Asn Gly
325 330 335

Val Trp Ile Gly Arg Thr Lys Ser Thr Asn Ser Arg Ser Gly Phe Glu
340 345 350

Met Ile Trp Asp Pro Asn Gly Trp Thr Gly Thr Asp Ser Ser Phe Ser
355 360 365

Val Lys Gln Asp Ile Val Ala Ile Thr Asp Trp Ser Gly Tyr Ser Gly
370 375 380

Ser Phe Val Gln His Pro Glu Leu Thr Gly Leu Asp Cys Ile Arg Pro
385 390 395 400

Cys Phe Trp Val Glu Leu Ile Arg Gly Arg Pro Lys Glu Ser Thr Ile
405 410 415

Trp Thr Ser Gly Ser Ser Ile Ser Phe Cys Gly Val Asn Ser Asp Thr
420 425 430

Val Ser Trp Ser Trp Pro Asp Gly Ala Glu Leu Pro Phe Thr Ile Asp
435 440 445

Lys

<210> 32
<211> 252
<212> PRT
<213> Unknown

<220>
<223> Vac2-mc-opt

<400> 32

Met Ser Leu Leu Thr Glu Val Glu Thr Tyr Val Leu Ser Ile Ile Pro
1 5 10 15

Ser Gly Pro Leu Lys Ala Glu Ile Ala Gln Lys Leu Glu Asp Val Phe
20 25 30

Ala Gly Lys Asn Thr Asp Leu Glu Ala Leu Met Glu Trp Leu Lys Thr
35 40 45

Arg Pro Ile Leu Ser Pro Leu Thr Lys Gly Ile Leu Gly Phe Val Phe

50 55 60

Thr Leu Thr Val Pro Ser Glu Arg Gly Leu Gln Arg Arg Arg Phe Val
65 70 75 80

Gln Asn Ala Leu Asn Gly Asn Gly Asp Pro Asn Asn Met Asp Arg Ala
85 90 95

Val Lys Leu Tyr Lys Lys Leu Lys Arg Glu Ile Thr Phe His Gly Ala
100 105 110

Lys Glu Val Ser Leu Ser Tyr Ser Thr Gly Ala Leu Ala Ser Cys Met
115 120 125

Gly Leu Ile Tyr Asn Arg Met Gly Thr Val Thr Thr Glu Val Ala Phe
130 135 140

Gly Leu Val Cys Ala Thr Cys Glu Gln Ile Ala Asp Ser Gln His Arg
145 150 155 160

Ser His Arg Gln Met Ala Thr Ile Thr Asn Pro Leu Ile Arg His Glu
165 170 175

Asn Arg Met Val Leu Ala Ser Thr Thr Ala Lys Ala Met Glu Gln Met
180 185 190

Ala Gly Ser Ser Glu Gln Ala Ala Glu Ala Met Glu Val Ala Asn Gln
195 200 205

Ala Arg Gln Met Val Gln Ala Met Arg Thr Ile Gly Thr His Pro Asn
210 215 220

Ser Ser Ala Gly Leu Arg Asp Asn Leu Leu Glu Asn Leu Gln Ala Tyr
225 230 235 240

Gln Lys Arg Met Gly Val Gln Met Gln Arg Phe Lys
245 250

<210> 33
<211> 564
<212> PRT
<213> Unknown

<220>
<223> VN1203-ha-cs-opt

<400> 33

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Met Glu Lys Ile Val Leu Leu Phe Ala Ile Val Ser Leu Val Lys Ser
1               5                   10                  15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
            20                  25                  30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
        35                  40                  45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
    50                  55                  60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65                  70                  75                  80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
                85                  90                  95

Glu Lys Ala Asn Pro Ala Asn Asp Leu Cys Tyr Pro Gly Asp Phe Asn
            100                 105                 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
        115                 120                 125

Lys Ile Gln Ile Ile Pro Lys Asn Ser Trp Ser Ser His Glu Ala Ser
    130                 135                 140

Leu Gly Val Ser Ser Ala Cys Pro Tyr Gln Gly Lys Ser Ser Phe Phe
145                 150                 155                 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Asn Ala Tyr Pro Thr Ile
                165                 170                 175

Lys Arg Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
            180                 185                 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
        195                 200                 205

Asn Pro Thr Thr Tyr Ile Ser Val Gly Thr Ser Thr Leu Asn Gln Arg
    210                 215                 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Asn Gly
225                 230                 235                 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
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245 250 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
260 265 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
275 280 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
290 295 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305 310 315 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
325 330 335

Pro Gln Arg Glu Thr Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile
340 345 350

Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr Gly Tyr His His
355 360 365

Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys Glu Ser Thr Gln
370 375 380

Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser Ile Ile Asp Lys
385 390 395 400

Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe Asn Asn Leu Glu
405 410 415

Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp Gly Phe Leu Asp
420 425 430

Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met Glu Asn Glu Arg
435 440 445

Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu Tyr Asp Lys Val
450 455 460

Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly Asn Gly Cys Phe
465 470 475 480

Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu Ser Val Arg Asn
485 490 495

Gly Thr Tyr Asp Tyr Pro Gln Tyr Ser Glu Glu Ala Arg Leu Lys Arg
500 505 510

Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly Thr Tyr Gln Ile
515 520 525

Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala Leu Ala Ile Met
530 535 540

Val Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly Ser Leu Gln Cys
545 550 555 560

Arg Ile Cys Ile

<210> 34
<211> 572
<212> PRT
<213> Unknown

<220>
<223> VN1203-ha-spc-opt

<400> 34

Met Pro Leu Tyr Lys Leu Leu Asn Val Leu Trp Leu Val Ala Val Ser
1 5 10 15

Asn Ala Ile Pro Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser
20 25 30

Thr Glu Gln Val Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His
35 40 45

Ala Gln Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu
50 55 60

Asp Gly Val Lys Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp
65 70 75 80

Leu Leu Gly Asn Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp
85 90 95

Ser Tyr Ile Val Glu Lys Ala Asn Pro Ala Asn Asp Leu Cys Tyr Pro
100 105 110

Gly Asp Phe Asn Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile

115 120 125

Asn His Phe Glu Lys Ile Gln Ile Ile Pro Lys Asn Ser Trp Ser Ser
130 135 140

His Glu Ala Ser Leu Gly Val Ser Ser Ala Cys Pro Tyr Gln Gly Lys
145 150 155 160

Ser Ser Phe Phe Arg Asn Val Val Trp Leu Ile Lys Lys Asn Asn Ala
165 170 175

Tyr Pro Thr Ile Lys Arg Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu
180 185 190

Leu Val Leu Trp Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr
195 200 205

Arg Leu Tyr Gln Asn Pro Thr Thr Tyr Ile Ser Val Gly Thr Ser Thr
210 215 220

Leu Asn Gln Arg Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn
225 230 235 240

Gly Gln Asn Gly Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn
245 250 255

Asp Ala Ile Asn Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr
260 265 270

Ala Tyr Lys Ile Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu
275 280 285

Leu Glu Tyr Gly Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala
290 295 300

Ile Asn Ser Ser Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly
305 310 315 320

Glu Cys Pro Lys Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly
325 330 335

Leu Arg Asn Ser Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg Gly Leu
340 345 350

Phe Gly Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val
355 360 365

Asp Gly Trp Tyr Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr
    370                 375                 380

Ala Ala Asp Lys Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn
385                 390                 395                 400

Lys Val Asn Ser Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val
                405                 410                 415

Gly Arg Glu Phe Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys
            420                 425                 430

Lys Met Glu Asp Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu
        435                 440                 445

Leu Val Leu Met Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn
    450                 455                 460

Val Lys Asn Leu Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala
465                 470                 475                 480

Lys Glu Leu Gly Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn
                485                 490                 495

Glu Cys Met Glu Ser Val Arg Asn Gly Thr Tyr Asp Tyr Pro Gln Tyr
            500                 505                 510

Ser Glu Glu Ala Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu
        515                 520                 525

Glu Ser Ile Gly Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala
    530                 535                 540

Ser Ser Leu Ala Leu Ala Ile Met Val Ala Gly Leu Ser Leu Trp Met
545                 550                 555                 560

Cys Ser Asn Gly Ser Leu Gln Cys Arg Ile Cys Ile
                565                 570

<210> 35
<211> 570
<212> PRT
<213> Unknown

<220>
<223> VN1203-ha-sph9-opt

<400> 35

Met Glu Thr Ile Ser Leu Ile Thr Ile Leu Leu Val Val Thr Ala Ser
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Asn Ala Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu
20 25 30

Gln Val Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln
35 40 45

Asp Ile Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly
50 55 60

Val Lys Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu
65 70 75 80

Gly Asn Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr
85 90 95

Ile Val Glu Lys Ala Asn Pro Ala Asn Asp Leu Cys Tyr Pro Gly Asp
100 105 110

Phe Asn Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His
115 120 125

Phe Glu Lys Ile Gln Ile Ile Pro Lys Asn Ser Trp Ser Ser His Glu
130 135 140

Ala Ser Leu Gly Val Ser Ser Ala Cys Pro Tyr Gln Gly Lys Ser Ser
145 150 155 160

Phe Phe Arg Asn Val Val Trp Leu Ile Lys Lys Asn Asn Ala Tyr Pro
165 170 175

Thr Ile Lys Arg Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val
180 185 190

Leu Trp Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu
195 200 205

Tyr Gln Asn Pro Thr Thr Tyr Ile Ser Val Gly Thr Ser Thr Leu Asn
210 215 220

Gln Arg Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln
225 230 235 240

Asn Gly Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala
245 250 255

Ile Asn Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr
260 265 270

Lys Ile Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu
275 280 285

Tyr Gly Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn
290 295 300

Ser Ser Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys
305 310 315 320

Pro Lys Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg
325 330 335

Asn Ser Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg Gly Leu Phe Gly
340 345 350

Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly
355 360 365

Trp Tyr Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala
370 375 380

Asp Lys Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn Lys Val
385 390 395 400

Asn Ser Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val Gly Arg
405 410 415

Glu Phe Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys Lys Met
420 425 430

Glu Asp Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu Leu Val
435 440 445

Leu Met Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn Val Lys
450 455 460

Asn Leu Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu
465 470 475 480

Leu Gly Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn Glu Cys
485 490 495

Met Glu Ser Val Arg Asn Gly Thr Tyr Asp Tyr Pro Gln Tyr Ser Glu
500 505 510

Glu Ala Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu Glu Ser
515 520 525

Ile Gly Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser
530 535 540

Leu Ala Leu Ala Ile Met Val Ala Gly Leu Ser Leu Trp Met Cys Ser
545 550 555 560

Asn Gly Ser Leu Gln Cys Arg Ile Cys Ile
565 570

<210> 36
<211> 1707
<212> DNA
<213> Influenza virus

<400> 36
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attggttacc atgcaaacaa ctcgacagag caggttgaca caataatgga aaagaacgtt 120

actgttacac atgcccaaga catactggaa aagaaacaca acgggaagct ctgcgatcta 180

gatggagtga agcctctaat tttgagagat tgtagcgtag ctggatggct cctcggaaac 240

ccaatgtgtg acgaattcat caatgtgccg gaatggtctt acatagtgga gaaggccaat 300

ccagtcaatg acctctgtta cccaggggat ttcaatgact atgaagaatt gaaacaccta 360

ttgagcagaa taaaccattt tgagaaaatt cagatcatcc ccaaaagttc ttggtccagt 420

catgaagcct cattaggggt gagctcagca tgtccatacc agggaaagtc ctcctttttc 480

agaaatgtgg tatggcttat caaaaagaac agtacatacc caacaataaa gaggagctac 540

aataatacca accaagaaga tcttttggta ctgtggggga ttcaccatcc taatgatgcg 600

gcagagcaga caaagctcta tcaaaaccca accacctata tttccgttgg gacatcaaca 660

ctaaaccaga gattggtacc aagaatagct actagatcca aagtaaacgg gcaaagtgga 720

aggatggagt tcttctggac aattttaaag ccgaatgatg caatcaactt cgagagtaat 780

ggaaatttca ttgctccaga atatgcatac aaaattgtca agaaagggga ctcaacaatt 840

atgaaaagtg aattggaata tggtaactgc aacaccaagt gtcaaactcc aatgggggcg 900 ataaactcta gcatgccatt ccacaatata caccctctca ccattgggga atgccccaaa 960

tatgtgaaat caaacagatt agtccttgcg actgggctca gaaatagccc tcaaagagag 1020

agaagaagaa aaaagagagg attatttgga gctatagcag gttttataga gggaggatgg 1080

cagggaatgg tagatggttg gtatgggtac caccatagca atgagcaggg gagtgggtac 1140

gctgcagaca aagaatccac tcaaaaggca atagatggag tcaccaataa ggtcaactcg 1200

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aggagaatag agaatttaaa caagaagatg gaagacgggt tcctagatgt ctggacttat 1320

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gtcaagaacc tttacgacaa ggtccgacta cagcttaggg ataatgcaaa ggagctgggt 1440

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ggagtaaaat tggaatcaat aggaatttac caaatactgt caatttattc tacagtggcg 1620

agttccctag cactggcaat catggtagct ggtctatcct tatggatgtg ctccaatgga 1680

tcgttacaat gcagaatttg catttaa 1707

<210> 37
<211> 1750
<212> DNA
<213> Influenza virus

<400> 37
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ttttgcaata gtcagtcttg ttaaaagtga tcagatttgc attggttacc atgcaaacaa 120

ctcgacagag caggttgaca caataatgga aaagaacgtt actgttacac atgcccaaga 180

catactggaa aagaaacaca acgggaagct ctgcgatcta gatggagtga agcctctaat 240

tttgagagat tgtagcgtag ctggatggct cctcggaaac ccaatgtgtg acgaattcat 300

caatgtgccg gaatggtctt acatagtgga gaaggccaat ccagtcaatg acctctgtta 360

cccaggggat ttcaatgact atgaagaatt gaaacaccta ttgagcagaa taaaccattt 420

tgagaaaatt cagatcatcc ccaaaagttc ttggtccagt catgaagcct cattaggggt 480

gagctcagca tgtccatacc agggaaagtc ctcctttttc agaaatgtgg tatggcttat 540

caaaaagaac agtacatacc caacaataaa gaggagctac aataatacca accaagaaga 600

tcttttggta ctgtggggga ttcaccatcc taatgatgcg gcagagcaga caaagctcta 660

tcaaaaccca accacctata tttccgttgg gacatcaaca ctaaaccaga gattggtacc 720

aagaatagct actagatcca aagtaaacgg gcaaagtgga aggatggagt tcttctggac 780 aattttaaag ccgaatgatg caatcaactt cgagagtaat ggaaatttca ttgctccaga 840

atatgcatac aaaattgtca agaaagggga ctcaacaatt atgaaaagtg aattggaata 900

tggtaactgc aacaccaagt gtcaaactcc aatgggggcg ataaactcta gcatgccatt 960

ccacaatata caccctctca ccattgggga atgccccaaa tatgtgaaat caaacagatt 1020

agtccttgcg actgggctca gaaatagccc tcaaagagag agaagaagaa aaaagagagg 1080

attatttgga gctatagcag gttttataga gggaggatgg cagggaatgg tagatggttg 1140

gtatgggtac caccatagca atgagcaggg gagtgggtac gctgcagaca aagaatccac 1200

tcaaaaggca atagatggag tcaccaataa ggtcaactcg atcattgaca aaatgaacac 1260

tcagtttgag gccgttggaa gggaatttaa caacttagaa aggagaatag agaatttaaa 1320

caagaagatg gaagacgggt tcctagatgt ctggacttat aatgctgaac ttctggttct 1380

catggaaaat gagagaactc tagactttca tgactcaaat gtcaagaacc tttacgacaa 1440

ggtccgacta cagcttaggg ataatgcaaa ggagctgggt aacggttgtt tcgagttcta 1500

tcataaatgt gataatgaat gtatggaaag tgtaagaaat ggaacgtatg actacccgca 1560

gtattcagaa gaagcgagac taaaaagaga ggaaataagt ggagtaaaat tggaatcaat 1620

aggaatttac caaatactgt caatttattc tacagtggcg agttccctag cactggcaat 1680

catggtagct ggtctatcct tatggatgtg ctccaatggg tcgttacaat gcagaatttg 1740

catttaagcg 1750

<210> 38
<211> 1350
<212> DNA
<213> Influenza virus

<400> 38
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agcttaatgt tacaaattgg gaacatgatc tcaatatggg tcagtcattc aattcacaca 120

gggaatcaac accaatctga accaatcagc aatactaatt ttcttactga gaaagctgtg 180

gcttcagtaa aattagcggg caattcatct ctttgcccca ttaacggatg ggctgtatac 240

agtaaggaca acagtataag gatcggttcc aaggggggatg tgtttgttat aagagagccg 300

ttcatctcat gctcccactt ggaatgcaga actttctttt tgactcaggg agccttgctg 360

aatgacaagc actccaatgg gactgtcaaa gacagaagcc ctcacagaac attaatgagt 420

tgtcctgtgg gtgaggctcc ctccccatat aactcaaggt ttgagtctgt tgcttggtca 480

gcaagtgctt gccatgatgg caccagttgg ttgacgattg gaatttctgg cccagacaat 540

ggggctgtgg ctgtattgaa atacaatggc ataataacag acactatcaa gagttggagg 600 aacaacatac tgagaactca agagtctgaa tgtgcatgtg taaatggctc ttgctttact 660

gtaatgactg acggaccaag taatggtcag gcatcacata agatcttcaa aatggaaaaa 720

gggaaagtgg ttaaatcagt cgaattggat gctcctaatt atcactatga ggaatgctcc 780

tgttatccta atgccggaga aatcacatgt gtgtgcaggg ataattggca tggctcaaat 840

cggccatggg tatctttcaa tcaaaatttg gagtatcaaa taggatatat atgcagtgga 900

gttttcggag acaatccacg ccccaatgat ggaacaggta gttgtggtcc ggtgtcctct 960

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agaaccaaaa gcactaattc caggagcggc tttgaaatga tttgggatcc aaatgggtgg 1080

actgaaacgg acagtagctt ttcagtgaaa caagatatcg tagcaataac tgattggtca 1140

ggatatagcg ggagttttgt ccagcatcca gaactgacag gactagattg cataagacct 1200

tgtttctggg ttgagttgat cagagggcgg cccaaagaga gcacaatttg gactagtggg 1260

agcagcatat ctttttgtgg tgtaaatagt gacactgtgg gttggtcttg gccagacggt 1320

gccgagttgc cattcaccat tgacaagtag 1350

<210> 39
<211> 1400
<212> DNA
<213> Influenza virus

<400> 39
ccgggatgaa tccaaatcag aagataataa ccatcggatc aatctgtatg gtaactggaa 60

tagttagctt aatgttacaa attgggaaca tgatctcaat atgggtcagt cattcaattc 120

acacagggaa tcaacaccaa tctgaaccaa tcagcaatac taattttctt actgagaaag 180

ctgtggcttc agtaaaatta gcgggcaatt catctctttg ccccattaac ggatgggctg 240

tatacagtaa ggacaacagt ataaggatcg gttccaaggg ggatgtgttt gttataagag 300

agccgttcat ctcatgctcc cacttggaat gcagaacttt ctttttgact cagggagcct 360

cgctgaatga caagcactcc aatgggactg tcaaagacag aagccctcac agaacattaa 420

tgagttgtcc tgtgggtgag gctccctccc catataactc aaggtttgag tctgttgctt 480

ggtcagcaag tgcttgccat gatggcacca gttggttgac gattggaatt tctggcccag 540

acaatggggc tgtggctgta ttgaaataca atggcataat aacagacact atcaagagtt 600

ggaggaacaa catactgaga actcaagagt ctgaatgtgc atgtgtaaat ggctcttgct 660

ttactgtaat gactgacgga ccaagtaatg gtcaggcatc acataagatc ttcaaaatgg 720

aaaaagggaa agtggttaaa tcagtcgaat tggatgctcc taattatcac tatgaggaat 780

gctcctgtta tcctaatgcc ggagaaatca catgtgtgtg cagggataat tggcatggct 840 caaatcggcc atgggtatct ttcaatcaaa atttggagta tcaaatagga tatatatgca 900

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cctctaacgg ggcatatggg gtaaaagggt tttcatttaa atacggcaat ggtgtctgga 1020

tcgggagaac caaaagcact aattccagga gcggctttga aatgatttgg gatccaaatg 1080

ggtggactga aacggacagt agcttttcag tgaaacaaga tatcgtagca ataactgatt 1140

ggtcaggata tagcgggagt tttgtccagc atccagaact gacaggacta gattgcataa 1200

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acggtgctga gttgccattc accattgaca agtaggggcc ctcgagtaag ggcgaattcc 1380

agcacactgg cggccgttac 1400

<210> 40
<211> 759
<212> DNA
<213> Influenza virus

<400> 40
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aaagccgaga tcgcacagaa acttgaagat gtctttgcag gaaagaacac cgatctcgag 120

gctctcatgg agtggctaaa gacaagacca atcctgtcac ctctgactaa agggattttg 180

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cagaatgccc taaatggaaa tggagatcca aataatatgg atagggcagt taagctatat 300

aagaagctga aaagagaaat aacattccat ggggctaagg aggtcgcact cagctactca 360

accggtgcac ttgccagttg catgggtctc atatacaaca ggatgggaac ggtgactacg 420

gaagtggctt ttggcctagt gtgtgccact tgtgagcaga ttgcagattc acagcatcgg 480

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gaagccatgg agatcgctaa tcaggctagg cagatggtgc aggcaatgag gacaattggg 660

actcatccta actctagtgc tggtctgaga gataatcttc ttgaaaattt gcaggcctac 720

cagaaacgaa tgggagtgca gatgcagcga ttcaagtga 759

<210> 41
<211> 793
<212> DNA
<213> Influenza virus

<400> 41
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gtacgttctc tctatcatcc cgtcaggccc cctcaaagcc gagatcgcac agaaacttga 120

agatgtcttt gcaggaaaga acaccgatct cgaggctctc atggagtggc taaagacaag 180

accaatcctg tcacctctga ctaaagggat tttgggattt gtattcacgc tcaccgtgcc 240

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tccaaataat atggataggg cagttaagct atataagaag ctgaaaagag aaataacatt 360

ccatggggct aaggaggtcg cactcagcta ctcaaccggt gcacttgcca gttgcatggg 420

tctcatatac aacaggatgg gaacggtgac tacggaagtg gcttttggcc tagtgtgtgc 480

cacttgtgag cagattgcag attcacagca tcggtctcac agacagatgg caactatcac 540

caacccacta atcagacatg agaacagaat ggtgctggcc agcactacag ctaaggctat 600

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taggcagatg gtgcaggcaa tgaggacaat tgggactcat cctaactcta gtgctggtct 720

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gcgattcaag tga 793

<210> 42
<211> 1740
<212> DNA
<213> Artificial sequence

<220>
<223> Influenza HA gene optimized for expression in insect cell expression system

<400> 42
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aagtccgacc agatctgcat cggttaccac gctaacaact ccaccgagca ggtggacacc 120

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gaggagctga agcacctgct gtcccgtatc aaccacttcg agaagatcca gatcatcccc 420

aagtcctctt ggtccgacca cgaggcttcc tccggtgtct cctccgcttg cccctacctg 480

ggttccccct ccttcttccg taacgtggtg tggctgatca agaagaactc cacctacccc 540

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cagaccccca tgggtgctat caactcctcc atgcccttcc acaacatcca cccctgacc 960

atcggcgagt gccccaagta cgtgaagtcc aaccgtctgg tgctggctac cggtctgcgt 1020

aactcccccc agcgcgagtc ccgtcgtaag aagcgtggtc tgttcggcgc tatcgctggt 1080

ttcatcgagg gcggttggca gggcatggtg gacggatggt acggttacca ccactctaac 1140

gagcagggtt ccggttacgc tgctgacaag gagtccaccc agaaggctat cgacggcgtc 1200

accaacaagg tgaactccat catcgacaag atgaacaccc agttcgaggc tgtgggtcgt 1260

gagttcaaca acctcgagcg tcgtatcgag aacctgaaca agaagatgga ggacggtttc 1320

ctggacgtgt ggacctacaa cgccgagctg ctggtgctga tggagaacga gcgtaccctg 1380

gacttccacg actccaacgt gaagaacctg tacgacaagg tccgcctgca gctgcgtgac 1440

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tggatgtgct ccaacggttc cctgcagtgc cgtatctgca tctaatgaaa gcttgagctc 1740

<210> 43
<211> 568
<212> PRT
<213> Influenza virus

<400> 43

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
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Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
            20                  25                  30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
        35                  40                  45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
    50                  55                  60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65                  70                  75                  80

```
Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
                85                  90                  95

Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser Phe Asn
            100                 105                 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
        115                 120                 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
    130                 135                 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser Phe Phe
145                 150                 155                 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro Thr Ile
                165                 170                 175

Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
            180                 185                 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
        195                 200                 205

Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn Gln Arg
    210                 215                 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225                 230                 235                 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
                245                 250                 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
            260                 265                 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
        275                 280                 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
    290                 295                 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305                 310                 315                 320
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Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
325 330 335

Pro Gln Arg Glu Ser Arg Arg Lys Lys Arg Gly Leu Phe Gly Ala Ile
340 345 350

Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr
355 360 365

Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys
370 375 380

Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser
385 390 395 400

Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe
405 410 415

Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp
420 425 430

Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met
435 440 445

Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu
450 455 460

Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly
465 470 475 480

Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu
485 490 495

Ser Ile Arg Asn Gly Thr Tyr Asn Tyr Pro Gln Tyr Ser Glu Glu Ala
500 505 510

Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly
515 520 525

Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala
530 535 540

Leu Ala Ile Met Met Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly
545 550 555 560

Ser Leu Gln Cys Arg Ile Cys Ile
565

<210> 44
<211> 1716
<212> DNA
<213> Artificial Sequence

<220>
<223> Influenza HA gene optimized for expression in insect cell expression system

<400> 44
ggatccgcca ccatggagaa gatcgtgctg ctgctggcta tcgtgtccct ggtgaagtcc 60

gaccagatct gcatcggtta ccacgctaac aactccaccg agcaggtgga caccatcatg 120

gagaagaacg tcaccgtgac ccacgctcag gacatcctcg aaaagaccca caacggcaag 180

ctgtgcgacc tggacggtgt caagcccctg atcctgcgtg actgctccgt ggctggttgg 240

ctgctgggta accccatgtg cgacgagttc atcaacgtgc ccgagtggtc ctacatcgtg 300

gagaaggcta accccaccaa cgacctgtgc taccccggtt ccttcaacga ctacgaggag 360

ctgaagcacc tgctgtcccg tatcaaccac ttcgagaaga tccagatcat ccccaagtcc 420

tcttggtccg accacgaggc ttcctccggt gtctcctccg cttgccccta cctgggttcc 480

ccctccttct tccgtaacgt ggtgtggctg atcaagaaga actccaccta ccccaccatc 540

aagaagtcct acaacaacac caaccaggag gacctgctgg tcctgtgggg tatccaccac 600

cccaacgacg ctgccgagca gacccgtctg taccagaacc ccaccaccta catctccatc 660

ggcacctcca ccctgaacca gcgtctggtg cccaagatcg ctacccgttc caaggtgaac 720

ggccagtccg gtcgtatgga gttcttctgg accatcctga agcctaacga cgctatcaac 780

ttcgagtcca acggcaactt catcgctccc gagtacgctt acaagatcgt gaagaagggc 840

gactccgcta tcatgaagtc cgagctggag tacggtaact gcaacaccaa gtgccagacc 900

cccatgggtg ctatcaactc ctccatgccc ttccacaaca tccacccccct gaccatcggc 960

gagtgcccca agtacgtgaa gtccaaccgt ctggtgctgg ctaccggtct gcgtaactcc 1020

ccccagcgcg agtcccgtgg tctgttcggc gctatcgctg gtttcatcga gggcggttgg 1080

cagggcatgg tggacggatg gtacggttac caccactcta acgagcaggg ttccggttac 1140

gctgctgaca aggagtccac ccagaaggct atcgacggcg tcaccaacaa ggtgaactcc 1200

atcatcgaca agatgaacac ccagttcgag gctgtgggtc gtgagttcaa caacctcgag 1260

cgtcgtatcg agaacctgaa caagaagatg gaggacggtt tcctggacgt gtggacctac 1320

aacgccgagc tgctggtgct gatggagaac gagcgtaccc tggacttcca cgactccaac 1380

gtgaagaacc tgtacgacaa ggtccgcctg cagctgcgtg acaacgctaa ggagctgggt 1440 aacggttgct tcgagttcta ccacaagtgc gacaacgagt gcatggagtc catccgtaac 1500

ggcacctaca actacccca gtactccgag gaggctcgtc tgaagcgtga ggagatctcc 1560

ggcgtgaagc tcgagtccat cggaacctac cagatcctgt ccatctactc caccgtggct 1620

tcctccctgg ctctggctat catgatggct ggtctgtccc tgtggatgtg ctccaacggt 1680

tccctgcagt gccgtatctg catctaatga aagctt 1716

<210> 45
<211> 564
<212> PRT
<213> Influenza virus

<400> 45

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
35 40 45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
50 55 60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65 70 75 80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
85 90 95

Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser Phe Asn
100 105 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
115 120 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
130 135 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser Phe Phe
145 150 155 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro Thr Ile
165 170 175

Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
180 185 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
195 200 205

Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn Gln Arg
210 215 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225 230 235 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
245 250 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
260 265 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
275 280 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
290 295 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305 310 315 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
325 330 335

Pro Gln Arg Glu Ser Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile
340 345 350

Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr Gly Tyr His His
355 360 365

Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys Glu Ser Thr Gln
370 375 380

Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser Ile Ile Asp Lys
385 390 395 400

Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe Asn Asn Leu Glu
405 410 415

Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp Gly Phe Leu Asp
420 425 430

Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met Glu Asn Glu Arg
435 440 445

Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu Tyr Asp Lys Val
450 455 460

Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly Asn Gly Cys Phe
465 470 475 480

Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu Ser Ile Arg Asn
485 490 495

Gly Thr Tyr Asn Tyr Pro Gln Tyr Ser Glu Glu Ala Arg Leu Lys Arg
500 505 510

Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly Thr Tyr Gln Ile
515 520 525

Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala Leu Ala Ile Met
530 535 540

Met Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly Ser Leu Gln Cys
545 550 555 560

Arg Ile Cys Ile

<210> 46
<211> 1383
<212> DNA
<213> Artificial Sequence

<220>
<223> Influenza NA gene optimized for expression in insect cell expression system

<400> 46
ggtaccggat ccgccaccat gaaccccaac cagaagatca tcaccatcgg ctccatctgc 60

atggtgatcg gtatcgtgtc cctgatgctg cagatcggta acatgatctc catctgggtg 120

tcccactcca tccagaccgg taaccagcac caggctgagt ccatctccaa caccaacccc 180

ctgaccgaga aggctgtggc ttccgtgacc ctggctggta actcctccct gtgccccatc 240

cgtggttggg ctgtgcactc caaggacaac aacatccgca tcggttccaa gggtgacgtg 300 ttcgtgatcc gtgagccctt catctcctgc tcccacctcg agtgccgtac cttcttcctg 360

acccaaggtg ctctgctgaa cgacaagcac tccaacggca ccgtgaagga ccgttccccc 420

caccgtaccc tgatgtcctg ccccgtgggc gaggctccct cccccctacaa ctcccgtttc 480

gagtccgtgg cttggtccgc ttccgcttgc cacgacggca cctcttggct gaccatcggt 540

atctccggtc ccgacaacga ggctgtcgct gtgctgaagt acaacggcat catcaccgac 600

accatcaagt cctggcgtaa caacatcctg cgtacccagg agtccgagtg cgcttgcgtg 660

aacggttcct gcttcaccgt gatgaccgac ggtccctccg acggccaggc ttcctacaag 720

atcttcaaga tggagaaggg caaggtggtg aagtccgtgg agctggacgc tcccaactac 780

cactacgagg agtgctcttg ctaccccgac gctggcgaga tcacctgcgt gtgccgtgac 840

aactggcacg gttccaaccg tccctgggtg tccttcaacc agaacctcga gtaccagatc 900

ggttacatct gctccggcgt gttcggtgac aacccccgtc ccaacgacgg aaccggttcc 960

tgcggtccca tgtcccccaa cggtgcttac ggtgtcaagg gcttctcctt caagtacggt 1020

aacggtgtct ggatcggtcg taccaagtcc accaactccc gctccggttt cgagatgatc 1080

tgggacccca acggttggac cggcaccgac tcttccttct ccgtgaagca ggacatcgtg 1140

gctatcaccg actggtccgg ttactccggt tccttcgtgc agcaccccga gctgaccggt 1200

ctggactgca ttcgtccctg cttctgggtg gagctgatcc gtggtcgtcc caaggagtcc 1260

accatctgga cctccggctc ctccatctct ttctgcggtg tgaactccga caccgtgtcc 1320

tggtcctggc ccgacggtgc cgagctgccc ttcaccatcg acaagtaatg aaagcttgag 1380

ctc 1383

<210> 47
<211> 449
<212> PRT
<213> Influenza virus

<400> 47

Met Asn Pro Asn Gln Lys Ile Ile Thr Ile Gly Ser Ile Cys Met Val
1 5 10 15

Ile Gly Ile Val Ser Leu Met Leu Gln Ile Gly Asn Met Ile Ser Ile
20 25 30

Trp Val Ser His Ser Ile Gln Thr Gly Asn Gln His Gln Ala Glu Ser
35 40 45

Ile Ser Asn Thr Asn Pro Leu Thr Glu Lys Ala Val Ala Ser Val Thr
50 55 60

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Leu Ala Gly Asn Ser Ser Leu Cys Pro Ile Arg Gly Trp Ala Val His
65                  70                  75                  80

Ser Lys Asp Asn Asn Ile Arg Ile Gly Ser Lys Gly Asp Val Phe Val
                85                  90                  95

Ile Arg Glu Pro Phe Ile Ser Cys Ser His Leu Glu Cys Arg Thr Phe
            100                 105                 110

Phe Leu Thr Gln Gly Ala Leu Leu Asn Asp Lys His Ser Asn Gly Thr
        115                 120                 125

Val Lys Asp Arg Ser Pro His Arg Thr Leu Met Ser Cys Pro Val Gly
    130                 135                 140

Glu Ala Pro Ser Pro Tyr Asn Ser Arg Phe Glu Ser Val Ala Trp Ser
145                 150                 155                 160

Ala Ser Ala Cys His Asp Gly Thr Ser Trp Leu Thr Ile Gly Ile Ser
                165                 170                 175

Gly Pro Asp Asn Glu Ala Val Ala Val Leu Lys Tyr Asn Gly Ile Ile
            180                 185                 190

Thr Asp Thr Ile Lys Ser Trp Arg Asn Asn Ile Leu Arg Thr Gln Glu
        195                 200                 205

Ser Glu Cys Ala Cys Val Asn Gly Ser Cys Phe Thr Val Met Thr Asp
    210                 215                 220

Gly Pro Ser Asp Gly Gln Ala Ser Tyr Lys Ile Phe Lys Met Glu Lys
225                 230                 235                 240

Gly Lys Val Val Lys Ser Val Glu Leu Asp Ala Pro Asn Tyr His Tyr
                245                 250                 255

Glu Glu Cys Ser Cys Tyr Pro Asp Ala Gly Glu Ile Thr Cys Val Cys
            260                 265                 270

Arg Asp Asn Trp His Gly Ser Asn Arg Pro Trp Val Ser Phe Asn Gln
        275                 280                 285

Asn Leu Glu Tyr Gln Ile Gly Tyr Ile Cys Ser Gly Val Phe Gly Asp
    290                 295                 300
```

Asn Pro Arg Pro Asn Asp Gly Thr Gly Ser Cys Gly Pro Met Ser Pro
305 310 315 320

Asn Gly Ala Tyr Gly Val Lys Gly Phe Ser Phe Lys Tyr Gly Asn Gly
325 330 335

Val Trp Ile Gly Arg Thr Lys Ser Thr Asn Ser Arg Ser Gly Phe Glu
340 345 350

Met Ile Trp Asp Pro Asn Gly Trp Thr Gly Thr Asp Ser Ser Phe Ser
355 360 365

Val Lys Gln Asp Ile Val Ala Ile Thr Asp Trp Ser Gly Tyr Ser Gly
370 375 380

Ser Phe Val Gln His Pro Glu Leu Thr Gly Leu Asp Cys Ile Arg Pro
385 390 395 400

Cys Phe Trp Val Glu Leu Ile Arg Gly Arg Pro Lys Glu Ser Thr Ile
405 410 415

Trp Thr Ser Gly Ser Ser Ile Ser Phe Cys Gly Val Asn Ser Asp Thr
420 425 430

Val Ser Trp Ser Trp Pro Asp Gly Ala Glu Leu Pro Phe Thr Ile Asp
435 440 445

Lys

<210> 48
<211> 792
<212> DNA
<213> Artificial Sequence

<220>
<223> Influenza M1 gene optimized for expression in insect cell expression system

<400> 48
ggtaccggat ccgccaccat gtccctgctg accgaggtgg agacctacgt gctgtccatc 60

atcccctccg gtcctctgaa ggctgagatc gctcagaagc tcgaggacgt tttcgctggc 120

aagaacaccg acctcgaggc tctgatggag tggctcaaga cccgtcccat cctgtccccc 180

ctgaccaagg gtatcctggg tttcgtgttc accctgaccg tgccctccga gcgtggtctg 240

cagcgtcgtc gtttcgtgca gaacgctctg aacggtaacg gtgaccccaa caacatggac 300

cgtgctgtga agctgtacaa gaagctgaag cgcgagatca ccttccacgg tgctaaggag 360 gtgtccctgt cctactccac cggtgctctg gctagctgca tgggcctgat ctacaaccgt 420

atgggcaccg tgaccaccga ggtggccttc ggtctggtct gcgctacctg cgagcagatc 480

gctgactccc agcaccgttc ccaccgtcag atggctacca tcaccaaccc cctgatccgt 540

cacgagaacc gtatggtgct ggcttccacc accgctaagg ctatggagca gatggctggt 600

tcctccgagc aggctgctga ggccatggag gtggccaacc aggctcgtca gatggtgcag 660

gctatgcgta ccatcggcac ccaccccaac tcctccgctg gtctgcgtga caacctgctc 720

gagaacctgc aggcttacca gaagcgtatg ggagtccaga tgcagcgctt caagtaatga 780

aagcttgagc tc 792

<210> 49
<211> 252
<212> PRT
<213> Influenza virus

<400> 49

Met Ser Leu Leu Thr Glu Val Glu Thr Tyr Val Leu Ser Ile Ile Pro
1 5 10 15

Ser Gly Pro Leu Lys Ala Glu Ile Ala Gln Lys Leu Glu Asp Val Phe
20 25 30

Ala Gly Lys Asn Thr Asp Leu Glu Ala Leu Met Glu Trp Leu Lys Thr
35 40 45

Arg Pro Ile Leu Ser Pro Leu Thr Lys Gly Ile Leu Gly Phe Val Phe
50 55 60

Thr Leu Thr Val Pro Ser Glu Arg Gly Leu Gln Arg Arg Arg Phe Val
65 70 75 80

Gln Asn Ala Leu Asn Gly Asn Gly Asp Pro Asn Asn Met Asp Arg Ala
85 90 95

Val Lys Leu Tyr Lys Lys Leu Lys Arg Glu Ile Thr Phe His Gly Ala
100 105 110

Lys Glu Val Ser Leu Ser Tyr Ser Thr Gly Ala Leu Ala Ser Cys Met
115 120 125

Gly Leu Ile Tyr Asn Arg Met Gly Thr Val Thr Thr Glu Val Ala Phe
130 135 140

Gly Leu Val Cys Ala Thr Cys Glu Gln Ile Ala Asp Ser Gln His Arg
145 150 155 160

Ser His Arg Gln Met Ala Thr Ile Thr Asn Pro Leu Ile Arg His Glu
165 170 175

Asn Arg Met Val Leu Ala Ser Thr Thr Ala Lys Ala Met Glu Gln Met
180 185 190

Ala Gly Ser Ser Glu Gln Ala Ala Glu Ala Met Glu Val Ala Asn Gln
195 200 205

Ala Arg Gln Met Val Gln Ala Met Arg Thr Ile Gly Thr His Pro Asn
210 215 220

Ser Ser Ala Gly Leu Arg Asp Asn Leu Leu Glu Asn Leu Gln Ala Tyr
225 230 235 240

Gln Lys Arg Met Gly Val Gln Met Gln Arg Phe Lys
245 250

<210> 50
<211> 1736
<212> DNA
<213> Artificial Sequence

<220>
<223> Influenza HA gene optimized for expression in insect cell expression system

<400> 50
ggtaccggat ccctcgagat ggagaagatc gtgctgctgc tggctatcgt gtccctggtg 60

aagtccgacc agatctgcat cggttaccac gctaacaact ccaccgagca ggtggacacc 120

atcatggaga agaacgtcac cgtgacccac gctcaggaca tcctggaaaa gacccacaac 180

ggcaagctgt gcgacctgga cggtgtcaag cccctgatcc tgcgtgactg ctccgtggct 240

ggttggctgc tgggtaaccc catgtgcgac gagttcatca acgtgcccga gtggtcctac 300

atcgtggaga aggctaaccc cgctaacgac ctgtgctacc ccggtaactt caacgactac 360

gaggagctga agcacctgct gtcccgtatc aaccacttcg agaagatcca gatcatcccc 420

aagtcctctt ggtccgacca cgaggcttcc tccggtgtct cctccgcttg cccataccag 480

ggcaccccat ctttcttccg taacgtggtg tggctgatca agaagaacaa cacctacccc 540

accatcaagc gttcctacaa caacaccaac caggaggacc tgctgatcct gtggggtatc 600

caccactcca acgacgctgc cgagcagacc aagctgtacc agaaccccac cacctacatc 660

tccgtgggca cctccaccct gaaccagcgt ctggtgccca agatcgctac ccgttccaag 720 gtgaacggcc agtccggtcg tatggacttc ttctggacca tcctgaagcc taacgacgct 780

atcaacttcg agtccaacgg caacttcatc gctcccgagt acgcttacaa gatcgtgaag 840

aagggcgact ccgctatcgt caagtccgag gtggagtacg gtaactgcaa caccaagtgc 900

cagaccccca tcggtgctat caactcctcc atgcccttcc acaacatcca cccccctgacc 960

atcggcgagt gccccaagta cgtgaagtcc aacaagctgg tgctggctac cggtctgcgt 1020

aactcccccc tgcgtgagcg tggtctgttc ggcgctatcg ctggtttcat cgagggcggt 1080

tggcagggca tggtggacgg ttggtacggt taccaccaca gcaacgagca gggttccggt 1140

tacgctgctg acaaggagtc cacccagaag gctatcgacg gcgtcaccaa caaggtgaac 1200

tccatcatcg acaagatgaa cacccagttc gaggctgtgg gtcgtgagtt caacaacctg 1260

gagcgtcgta tcgagaacct gaacaagaag atggaggacg gtttcctgga cgtgtggacc 1320

tacaacgccg agctgctggt gctgatggag aacgagcgta ccctggactt ccacgactct 1380

aacgtgaaga acctgtacga caaggtccgc ctgcagctgc gtgacaacgc taaggagctg 1440

ggtaacggtt gcttcgagtt ctaccacaag tgcgacaacg agtgcatgga gtccgtgcgt 1500

aacggcacct acgactaccc ccagtactcc gaggaggctc gtctgaagcg tgaggagatc 1560

tccggcgtga agctggagtc catcggcacc taccagatcc tgtccatcta ctccaccgtg 1620

gcttcctccc tggctctggc tatcatggtg gctggtctgt ccctgtggat gtgctccaac 1680

ggttccctgc agtgccgtat ctgcatctaa taatgaggcg cgccaagctt gagctc 1736

<210> 51
<211> 563
<212> PRT
<213> Influenza virus

<400> 51

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
35 40 45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
50 55 60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65 70 75 80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
85 90 95

Glu Lys Ala Asn Pro Ala Asn Asp Leu Cys Tyr Pro Gly Asn Phe Asn
100 105 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
115 120 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
130 135 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Gln Gly Thr Pro Ser Phe Phe
145 150 155 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Asn Thr Tyr Pro Thr Ile
165 170 175

Lys Arg Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Ile Leu Trp
180 185 190

Gly Ile His His Ser Asn Asp Ala Ala Glu Gln Thr Lys Leu Tyr Gln
195 200 205

Asn Pro Thr Thr Tyr Ile Ser Val Gly Thr Ser Thr Leu Asn Gln Arg
210 215 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225 230 235 240

Arg Met Asp Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
245 250 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
260 265 270

Val Lys Lys Gly Asp Ser Ala Ile Val Lys Ser Glu Val Glu Tyr Gly
275 280 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Ile Gly Ala Ile Asn Ser Ser
290 295 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305 310 315 320

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Tyr Val Lys Ser Asn Lys Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
                325                 330                 335

Pro Leu Arg Glu Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu
            340                 345                 350

Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr Gly Tyr His His Ser
        355                 360                 365

Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys Glu Ser Thr Gln Lys
    370                 375                 380

Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser Ile Ile Asp Lys Met
385                 390                 395                 400

Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe Asn Asn Leu Glu Arg
                405                 410                 415

Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp Gly Phe Leu Asp Val
            420                 425                 430

Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met Glu Asn Glu Arg Thr
        435                 440                 445

Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu Tyr Asp Lys Val Arg
    450                 455                 460

Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly Asn Gly Cys Phe Glu
465                 470                 475                 480

Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu Ser Val Arg Asn Gly
                485                 490                 495

Thr Tyr Asp Tyr Pro Gln Tyr Ser Glu Glu Ala Arg Leu Lys Arg Glu
            500                 505                 510

Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly Thr Tyr Gln Ile Leu
        515                 520                 525

Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala Leu Ala Ile Met Val
    530                 535                 540

Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly Ser Leu Gln Cys Arg
545                 550                 555                 560
```

Ile Cys Ile

<210> 52
<211> 1738
<212> DNA
<213> Artificial Sequence

<220>
<223> Influenza HA gene optimized for expression in insect cell expression system

<400> 52
cgggcgcgga gcggccgcat ggagaagatc gtgctgctgc tggctatcgt gtctctggtc 60

aagtccgacc agatctgcat cggttaccac gctaacaact ccaccgagca ggtggacacc 120

atcatggaga agaacgtcac cgtgacccac gctcaggaca tcctcgaaaa gacccacaac 180

ggcaagctgt gcgacctgga cggcgtgaag cccctgatcc tgcgtgactg ctccgtggct 240

ggttggctgc tgggtaaccc catgtgcgac gagttcctca acgtgcccga gtggtcctac 300

atcgtggaga agatcaaccc cgctaacgac ctgtgctacc ccggtaactt caacgactac 360

gaggagctga agcacctgct gtcccgtatc aaccacttcg agaagatcca gatcatcccc 420

aagtcctctt ggtccgacca cgaggcttcc tccggtgtct cctccgcttg cccataccag 480

ggccgttctt ccttcttccg caacgtggtg tggctgatca agaagaacaa cgcctacccc 540

accatcaagc gttcctacaa caacaccaac caggaggacc tgctggtcct gtggggtatc 600

caccacccca acgacgctgc cgagcagacc cgtctgtacc agaaccccac cacctacatc 660

tccgtgggca cctctaccct gaaccagcgt ctggtgccca agatcgctac ccgttccaag 720

gtgaacggcc agtccggtcg tatggagttc ttctggacca tcctgaagcc taacgacgct 780

atcaacttcg agtccaacgg caacttcatc gctcccgaga acgcttacaa gatcgtgaag 840

aagggcgact ccaccatcat gaagtccgag ctggagtacg gcaactgcaa cactaagtgc 900

cagaccccca tcggtgctat caactcctcc atgcccttcc acaacatcca cccccctgact 960

atcggcgagt gccccaagta cgtgaagtcc aaccgtctgg tgctggctac cggtctgcgt 1020

aactcccccc agatcgagac tcgtggtctg ttcggcgcta tcgctggttt catcgagggc 1080

ggttggcagg gcatggtgga cggttggtac ggttaccacc actctaacga gcagggttcc 1140

ggttacgctg ctgacaagga gtctacccag aaggctatcg acggcgtcac caacaaggtg 1200

aactccatca tcgacaagat gaacacccag ttcgaggctg tgggtcgtga gttcaacaac 1260

ctcgaacgtc gtatcgagaa cctgaacaag aagatggagg acggtttcct ggacgtgtgg 1320

acctacaacg ccgagctgct ggtgctgatg gagaacgagc gtaccctgga cttccacgac 1380

tccaacgtga agaacctgta cgacaaggtc cgcctgcagc tgcgtgacaa cgctaaggag 1440 ctgggtaacg gttgcttcga gttctaccac cgttgcgaca acgagtgcat ggagtccgtg 1500

cgtaacggca cctacgacta cccccagtac tccgaggagg ctcgtctgaa gcgtgaggag 1560

atctccggtg tcaagctcga atccatcgga acctaccaga tcctgtccat ctactccacc 1620

gtggcttcct ccctggctct ggctatcatg gtggctggtc tgtccctgtg gatgtgctcc 1680

aacggttccc tgcagtgccg tatctgcatc taataatgag gcgcgccaag cttgtcga 1738

<210> 53
<211> 564
<212> PRT
<213> Influenza virus

<400> 53

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
35 40 45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
50 55 60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65 70 75 80

Pro Met Cys Asp Glu Phe Leu Asn Val Pro Glu Trp Ser Tyr Ile Val
85 90 95

Glu Lys Ile Asn Pro Ala Asn Asp Leu Cys Tyr Pro Gly Asn Phe Asn
100 105 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
115 120 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
130 135 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Gln Gly Arg Ser Ser Phe Phe
145 150 155 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Asn Ala Tyr Pro Thr Ile
165 170 175

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Lys Arg Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
            180                 185                 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
        195                 200                 205

Asn Pro Thr Thr Tyr Ile Ser Val Gly Thr Ser Thr Leu Asn Gln Arg
    210                 215                 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225                 230                 235                 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
                245                 250                 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Asn Ala Tyr Lys Ile
            260                 265                 270

Val Lys Lys Gly Asp Ser Thr Ile Met Lys Ser Glu Leu Glu Tyr Gly
        275                 280                 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Ile Gly Ala Ile Asn Ser Ser
    290                 295                 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305                 310                 315                 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
                325                 330                 335

Pro Gln Ile Glu Thr Arg Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile
            340                 345                 350

Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr Gly Tyr His His
        355                 360                 365

Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys Glu Ser Thr Gln
    370                 375                 380

Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser Ile Ile Asp Lys
385                 390                 395                 400

Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe Asn Asn Leu Glu
                405                 410                 415
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Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp Gly Phe Leu Asp
420 425 430

Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met Glu Asn Glu Arg
435 440 445

Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu Tyr Asp Lys Val
450 455 460

Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly Asn Gly Cys Phe
465 470 475 480

Glu Phe Tyr His Arg Cys Asp Asn Glu Cys Met Glu Ser Val Arg Asn
485 490 495

Gly Thr Tyr Asp Tyr Pro Gln Tyr Ser Glu Glu Ala Arg Leu Lys Arg
500 505 510

Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly Thr Tyr Gln Ile
515 520 525

Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala Leu Ala Ile Met
530 535 540

Val Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly Ser Leu Gln Cys
545 550 555 560

Arg Ile Cys Ile

<210> 54
<211> 1422
<212> DNA
<213> Artificial Sequence

<220>
<223> Influenza NA gene optimized for expression in insect cell expression system

<400> 54
accgtcccac catcgggcgc ggatccctcg agatgaaccc caaccagaag atcatcacca 60

tcggctccat ctgcatggtg atcggtatcg tgtccctgat gctgcagatc ggtaacatga 120

tctccatctg ggtgtcccac tccatccaga ccggtaacca gcgtcaggcc gagcccatct 180

ccaacaccaa gttcctcacc gagaaggctg tggcttccgt gaccctggct ggtaactcct 240

ccctgtgccc catctccggt tgggctgtgt actccaagga caactccatc cgtatcggtt 300 cccgtggtga cgtgttcgtg atccgtgagc ccttcatctc ctgctcccac ctcgaatgcc 360

gtaccttctt cctgacccag ggtgctctgc tgaacgacaa gcactccaac ggcaccgtga 420

aggaccgttc cccccaccgt accctgatgt cctgccccgt gggcgaggct ccctccccct 480

acaactcccg tttcgagtcc gtggcttggt ccgcttccgc ttgccacgac ggcacctctt 540

ggctgaccat cggtatctcc ggtcccgaca acggtgctgt ggctgtgctg aagtacaacg 600

gcatcatcac cgacaccatc aagtcctggc gtaacaacat cctgcgtacc caagagtccg 660

agtgcgcttg cgtgaacggt tcctgcttca ccgtgatgac cgacggtccc tccaacggcc 720

aggcttccta caagatcttc aagatggaga agggcaaggt ggtgaagtcc gtggagctgg 780

acgctcccaa ctaccactac gaggagtgct cttgctaccc cgacgctggc gagatcacct 840

gcgtgtgccg tgacaactgg cacggttcca accgtccctg ggtgtccttc aaccagaacc 900

tcgaatacca gatcggttac atctgctccg gcgtgttcgg tgacaacccc cgtcccaacg 960

acggaaccgg ttcctgcggt cccgtgtccc ccaacggtgc ttacggtgtc aagggcttct 1020

ccttcaagta cggtaacggt gtctggatcg gtcgtaccaa gtccaccaac tcccgctccg 1080

gtttcgagat gatctgggac cccaacggtt ggaccggcac cgactcttcc ttctccgtga 1140

agcaggacat cgtggctatc accgactggt ccggttactc cggttccttc gtgcagcacc 1200

ccgagctgac cggtctggac tgtatccgtc cctgcttctg ggtggagctg atccgtggtc 1260

gtcccaagga gtccaccatc tggacctccg gctcctccat ctctttctgc ggtgtgaact 1320

ccgacaccgt gtcctggtcc tggcccgacg gtgccgagct gcccttcacc atcgacaagt 1380

aataatgaat cgatttgtcg agaagtacta gaggatcata at 1422

<210> 55
<211> 449
<212> PRT
<213> Influenza virus

<400> 55

Met Asn Pro Asn Gln Lys Ile Ile Thr Ile Gly Ser Ile Cys Met Val
1 5 10 15

Ile Gly Ile Val Ser Leu Met Leu Gln Ile Gly Asn Met Ile Ser Ile
20 25 30

Trp Val Ser His Ser Ile Gln Thr Gly Asn Gln Arg Gln Ala Glu Pro
35 40 45

Ile Ser Asn Thr Lys Phe Leu Thr Glu Lys Ala Val Ala Ser Val Thr
50 55 60

Leu Ala Gly Asn Ser Ser Leu Cys Pro Ile Ser Gly Trp Ala Val Tyr
65 70 75 80

Ser Lys Asp Asn Ser Ile Arg Ile Gly Ser Arg Gly Asp Val Phe Val
85 90 95

Ile Arg Glu Pro Phe Ile Ser Cys Ser His Leu Glu Cys Arg Thr Phe
100 105 110

Phe Leu Thr Gln Gly Ala Leu Leu Asn Asp Lys His Ser Asn Gly Thr
115 120 125

Val Lys Asp Arg Ser Pro His Arg Thr Leu Met Ser Cys Pro Val Gly
130 135 140

Glu Ala Pro Ser Pro Tyr Asn Ser Arg Phe Glu Ser Val Ala Trp Ser
145 150 155 160

Ala Ser Ala Cys His Asp Gly Thr Ser Trp Leu Thr Ile Gly Ile Ser
165 170 175

Gly Pro Asp Asn Gly Ala Val Ala Val Leu Lys Tyr Asn Gly Ile Ile
180 185 190

Thr Asp Thr Ile Lys Ser Trp Arg Asn Asn Ile Leu Arg Thr Gln Glu
195 200 205

Ser Glu Cys Ala Cys Val Asn Gly Ser Cys Phe Thr Val Met Thr Asp
210 215 220

Gly Pro Ser Asn Gly Gln Ala Ser Tyr Lys Ile Phe Lys Met Glu Lys
225 230 235 240

Gly Lys Val Val Lys Ser Val Glu Leu Asp Ala Pro Asn Tyr His Tyr
245 250 255

Glu Glu Cys Ser Cys Tyr Pro Asp Ala Gly Glu Ile Thr Cys Val Cys
260 265 270

Arg Asp Asn Trp His Gly Ser Asn Arg Pro Trp Val Ser Phe Asn Gln
275 280 285

Asn Leu Glu Tyr Gln Ile Gly Tyr Ile Cys Ser Gly Val Phe Gly Asp
290 295 300

Asn Pro Arg Pro Asn Asp Gly Thr Gly Ser Cys Gly Pro Val Ser Pro
305 310 315 320

Asn Gly Ala Tyr Gly Val Lys Gly Phe Ser Phe Lys Tyr Gly Asn Gly
325 330 335

Val Trp Ile Gly Arg Thr Lys Ser Thr Asn Ser Arg Ser Gly Phe Glu
340 345 350

Met Ile Trp Asp Pro Asn Gly Trp Thr Gly Thr Asp Ser Ser Phe Ser
355 360 365

Val Lys Gln Asp Ile Val Ala Ile Thr Asp Trp Ser Gly Tyr Ser Gly
370 375 380

Ser Phe Val Gln His Pro Glu Leu Thr Gly Leu Asp Cys Ile Arg Pro
385 390 395 400

Cys Phe Trp Val Glu Leu Ile Arg Gly Arg Pro Lys Glu Ser Thr Ile
405 410 415

Trp Thr Ser Gly Ser Ser Ile Ser Phe Cys Gly Val Asn Ser Asp Thr
420 425 430

Val Ser Trp Ser Trp Pro Asp Gly Ala Glu Leu Pro Phe Thr Ile Asp
435 440 445

Lys

<210> 56
<211> 1750
<212> DNA
<213> Influenza virus

<400> 56
attcgccctt aacggtccga tggagaaaat agtgcttctt cttgcaatag tcagtcttgt 60

taaaagtgat cagatttgca ttggttacca tgcaaacaat tcaacagagc aggttgacac 120

aatcatggaa aagaacgtta ctgttacaca tgcccaagac atactggaaa agacacacaa 180

cgggaagctc tgcgatctag atggagtgaa gcctctaatt ttaagagatt gtagtgtagc 240

tggatggctc ctcgggaacc caatgtgtga cgaattcatc aatgtaccgg aatggtctta 300

catagtggag aaggccaatc caaccaatga cctctgttac ccagggagtt tcaacgacta 360

tgaagaactg aaacacctat tgagcagaat aaaccatttt gagaaaattc aaatcatccc 420

caaaagttct tggtccgatc atgaagcctc atcaggagtg agctcagcat gtccatacct 480 gggaagtccc tcctttttta gaaatgtggt atggcttatc aaaaagaaca gtacataccc 540

aacaataaag aaaagctaca ataataccaa ccaagaagat cttttggtac tgtggggaat 600

tcaccatcct aatgatgcgg cagagcagac aaggctatat caaaacccaa ccacctatat 660

ttccattggg acatcaacac taaaccagag attggtacca aaaatagcta ctagatccaa 720

agtaaacggg caaagtggaa ggatggagtt cttctggaca attttaaaac ctaatgatgc 780

aatcaacttc gagagtaatg gaaatttcat tgctccagaa tatgcataca aaattgtcaa 840

gaaaggggac tcagcaatta tgaaaagtga attggaatat ggtaactgca acaccaagtg 900

tcaaactcca atgggggcga taaactctag tatgccattc cacaacatac accctctcac 960

catcggggaa tgccccaaat atgtgaaatc aaacagatta gtccttgcaa cagggctcag 1020

aaatagccct caaagagaga gcagaagaaa aaagagagga ctatttggag ctatagcagg 1080

ttttatagag ggaggatggc agggaatggt agatggttgg tatgggtacc accatagcaa 1140

tgagcagggg agtgggtacg ctgcagacaa agaatccact caaaaggcaa tggatggagt 1200

caccaataag gtcaactcaa tcattgacaa aatgaacact cagtttgagg ccgttggaag 1260

ggaatttaat aacttagaaa ggagaataga gaatttaaac aagaagatgg aagacgggtt 1320

tctagatgtc tggacttata atgccgaact tctggttctc atggaaaatg agagaactct 1380

agactttcat gactcaaatg ttaagaacct ctacgacaag gtccgactac agcttaggga 1440

taatgcaaag gagctgggta acggttgttt cgagttctat cacaaatgtg ataatgaatg 1500

tatggaaagt ataagaaacg gaacgtgcaa ctatccgcag tattcagaag aagcaagatt 1560

aaaaagagag gaaataagtg gggtaaaatt ggaatcaata ggaacttacc aaatactgtc 1620

aatttattca acagtggcga gttccctagc actggcaatc atgatggctg gtctatcttt 1680

atggatgtgc tccaatggat cgttacaatg cagaatttgc atttaaaagc tttaagggcg 1740

aattccagca 1750

<210> 57
<211> 568
<212> PRT
<213> Influenza virus

<400> 57

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

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Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
        35                  40                  45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
    50                  55                  60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65                  70                  75                  80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
                85                  90                  95

Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser Phe Asn
            100                 105                 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
        115                 120                 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
    130                 135                 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser Phe Phe
145                 150                 155                 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro Thr Ile
                165                 170                 175

Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
            180                 185                 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
        195                 200                 205

Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn Gln Arg
    210                 215                 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225                 230                 235                 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
                245                 250                 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
            260                 265                 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
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275 280 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
290 295 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305 310 315 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
325 330 335

Pro Gln Arg Glu Ser Arg Arg Lys Lys Arg Gly Leu Phe Gly Ala Ile
340 345 350

Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr
355 360 365

Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys
370 375 380

Glu Ser Thr Gln Lys Ala Met Asp Gly Val Thr Asn Lys Val Asn Ser
385 390 395 400

Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe
405 410 415

Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp
420 425 430

Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met
435 440 445

Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu
450 455 460

Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly
465 470 475 480

Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu
485 490 495

Ser Ile Arg Asn Gly Thr Cys Asn Tyr Pro Gln Tyr Ser Glu Glu Ala
500 505 510

Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly
515 520 525

Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala
530 535 540

Leu Ala Ile Met Met Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly
545 550 555 560

Ser Leu Gln Cys Arg Ile Cys Ile
565

<210> 58
<211> 568
<212> PRT
<213> Influenza virus

<400> 58

Met Glu Lys Ile Val Leu Leu Leu Ala Ile Val Ser Leu Val Lys Ser
1 5 10 15

Asp Gln Ile Cys Ile Gly Tyr His Ala Asn Asn Ser Thr Glu Gln Val
20 25 30

Asp Thr Ile Met Glu Lys Asn Val Thr Val Thr His Ala Gln Asp Ile
35 40 45

Leu Glu Lys Thr His Asn Gly Lys Leu Cys Asp Leu Asp Gly Val Lys
50 55 60

Pro Leu Ile Leu Arg Asp Cys Ser Val Ala Gly Trp Leu Leu Gly Asn
65 70 75 80

Pro Met Cys Asp Glu Phe Ile Asn Val Pro Glu Trp Ser Tyr Ile Val
85 90 95

Glu Lys Ala Asn Pro Thr Asn Asp Leu Cys Tyr Pro Gly Ser Phe Asn
100 105 110

Asp Tyr Glu Glu Leu Lys His Leu Leu Ser Arg Ile Asn His Phe Glu
115 120 125

Lys Ile Gln Ile Ile Pro Lys Ser Ser Trp Ser Asp His Glu Ala Ser
130 135 140

Ser Gly Val Ser Ser Ala Cys Pro Tyr Leu Gly Ser Pro Ser Phe Phe
145 150 155 160

Arg Asn Val Val Trp Leu Ile Lys Lys Asn Ser Thr Tyr Pro Thr Ile
165 170 175

Lys Lys Ser Tyr Asn Asn Thr Asn Gln Glu Asp Leu Leu Val Leu Trp
180 185 190

Gly Ile His His Pro Asn Asp Ala Ala Glu Gln Thr Arg Leu Tyr Gln
195 200 205

Asn Pro Thr Thr Tyr Ile Ser Ile Gly Thr Ser Thr Leu Asn Gln Arg
210 215 220

Leu Val Pro Lys Ile Ala Thr Arg Ser Lys Val Asn Gly Gln Ser Gly
225 230 235 240

Arg Met Glu Phe Phe Trp Thr Ile Leu Lys Pro Asn Asp Ala Ile Asn
245 250 255

Phe Glu Ser Asn Gly Asn Phe Ile Ala Pro Glu Tyr Ala Tyr Lys Ile
260 265 270

Val Lys Lys Gly Asp Ser Ala Ile Met Lys Ser Glu Leu Glu Tyr Gly
275 280 285

Asn Cys Asn Thr Lys Cys Gln Thr Pro Met Gly Ala Ile Asn Ser Ser
290 295 300

Met Pro Phe His Asn Ile His Pro Leu Thr Ile Gly Glu Cys Pro Lys
305 310 315 320

Tyr Val Lys Ser Asn Arg Leu Val Leu Ala Thr Gly Leu Arg Asn Ser
325 330 335

Pro Gln Arg Glu Ser Arg Arg Lys Lys Arg Gly Leu Phe Gly Ala Ile
340 345 350

Ala Gly Phe Ile Glu Gly Gly Trp Gln Gly Met Val Asp Gly Trp Tyr
355 360 365

Gly Tyr His His Ser Asn Glu Gln Gly Ser Gly Tyr Ala Ala Asp Lys
370 375 380

Glu Ser Thr Gln Lys Ala Ile Asp Gly Val Thr Asn Lys Val Asn Ser
385 390 395 400

Ile Ile Asp Lys Met Asn Thr Gln Phe Glu Ala Val Gly Arg Glu Phe

405 410 415

Asn Asn Leu Glu Arg Arg Ile Glu Asn Leu Asn Lys Lys Met Glu Asp
420 425 430

Gly Phe Leu Asp Val Trp Thr Tyr Asn Ala Glu Leu Leu Val Leu Met
435 440 445

Glu Asn Glu Arg Thr Leu Asp Phe His Asp Ser Asn Val Lys Asn Leu
450 455 460

Tyr Asp Lys Val Arg Leu Gln Leu Arg Asp Asn Ala Lys Glu Leu Gly
465 470 475 480

Asn Gly Cys Phe Glu Phe Tyr His Lys Cys Asp Asn Glu Cys Met Glu
485 490 495

Ser Ile Arg Asn Gly Thr Tyr Asn Tyr Pro Gln Tyr Ser Glu Glu Ala
500 505 510

Arg Leu Lys Arg Glu Glu Ile Ser Gly Val Lys Leu Glu Ser Ile Gly
515 520 525

Thr Tyr Gln Ile Leu Ser Ile Tyr Ser Thr Val Ala Ser Ser Leu Ala
530 535 540

Leu Ala Ile Met Met Ala Gly Leu Ser Leu Trp Met Cys Ser Asn Gly
545 550 555 560

Ser Leu Gln Cys Arg Ile Cys Ile
565