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(54) **Titre : ANTICORPS DIRIGES CONTRE L'HEMAGGLUTININE DU VIRUS DE LA GRIPPE, COMPOSITIONS, ET PROCEDES ASSOCIES**
(54) **Title: INFLUENZA HEMAGGLUTININ ANTIBODIES, COMPOSITIONS, AND RELATED METHODS**

(57) **Abrégé/Abstract:**
Antibodies against influenza hemagglutinin, compositions containing the antibodies, and methods of using the antibodies are provided herein.

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(54) Title: INFLUENZA HEMAGGLUTININ ANTIBODIES, COMPOSITIONS, AND RELATED METHODS

(57) Abstract: Antibodies against influenza hemagglutinin, compositions containing the antibodies, and methods of using the antibodies are provided herein.

INFLUENZA HEMAGGLUTININ ANTIBODIES, COMPOSITIONS, AND RELATED METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims benefit of priority from U.S. Provisional Application Serial No. 61/246,958, filed on September 29, 2009.

TECHNICAL FIELD

[002] This invention relates to influenza hemagglutinin antibodies, and to materials and methods for making and using influenza hemagglutinin antibodies.

BACKGROUND

[003] Influenza has a long history characterized by waves of pandemics, epidemics, resurgences and outbreaks. Influenza is a highly contagious disease that could be equally devastating both in developing and developed countries. The influenza virus presents one of the major threats to the human population. In spite of annual vaccination efforts, influenza infections result in substantial morbidity and mortality. Although flu epidemics occur nearly every year, fortunately pandemics do not occur very often. However, recent flu strains have emerged such that we are again faced with the potential of an influenza pandemic. Avian influenza virus of the type H5N1, currently causing an epidemic in poultry in Asia as well as regions of Eastern Europe, has persistently spread throughout the globe. The rapid spread of infection, as well as cross species transmission from birds to human subjects, increases the potential for outbreaks in human populations and the risk of a pandemic. The virus is highly pathogenic, resulting in a mortality rate of over fifty percent in birds as well as the few human cases which have been identified. If the virus were to achieve human to human transmission, it would have the potential to result in rapid, widespread illness and mortality.

[004] Subtypes of the influenza virus are designated by different HA and NA resulting from antigenic shift. Furthermore, new strains of the same subtype result from antigenic drift, or mutations in the HA or NA molecules which generate new and different epitopes. While technological advances have improved the ability to produce improved influenza antigens vaccine compositions, there remains a need to provide additional sources of protection against to address emerging subtypes and strains of influenza.

SUMMARY

[005] This document relates to antibody compositions and methods for producing antibody compositions, including production in plant systems. This document further relates to vectors encoding antibodies or antigen binding fragments thereof, as well as fusion proteins, plant cells, plants, compositions, and kits comprising antibodies or antigen binding fragments thereof, and therapeutic and diagnostic uses in association with influenza infection in a subject.

[006] This document is based in part on the identification of anti-H5N1 hemagglutinin monoclonal antibodies (mAbs) that specifically inhibit hemagglutination of highly pathogenic avian influenza (HPAI). The protective efficacy of one of these antibodies has been demonstrated in animal challenge models (*e.g.*, mouse models) using homologous virus. The specific and effective inhibition of these antibodies makes them useful as therapeutic tool in the treatment and/or prevention of human infection. In addition, the mAbs can be a useful diagnostic tool for typing suspected H5N1 human isolates in conjunction with other diagnostic approaches. Thus, this document provides antibodies against influenza hemagglutinin antigens, as well as antibody components produced in plants. The antibodies can inhibit hemagglutination. Also provided are antibody compositions that are reactive against influenza hemagglutinin antigen. In addition, methods for production and use of the antibodies and compositions are provided herein.

[007] Thus, in a first aspect, this document features an isolated monoclonal antibody that binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody is selected from the group consisting of an antibody comprising a light chain variable region amino acid sequence at least 85% identical to the amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79 and a heavy chain variable region amino acid sequence at least 85% identical to the amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78; and an antibody comprising a light chain variable region amino acid sequence at least 85% identical to the amino acid sequence as set forth in amino acids 1-96 of SEQ ID NO:81 and a heavy chain variable region amino acid sequence at least 85% identical to the amino acid sequence as set forth in amino acids 1-112 of SEQ ID NO:80.

[008] The antibody can have a light chain variable region amino acid sequence at least 90% identical to the amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79, and a heavy chain variable region amino acid sequence at least 90% identical to the amino acid

sequence as set forth in amino acids 1-115 of SEQ ID NO:78. The antibody can have a light chain variable region amino acid sequence at least 95% identical to the amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79, and a heavy chain variable region amino acid sequence at least 95% identical to the amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78. The antibody can have a light chain variable region amino acid sequence at least 98% identical to the amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79, and a heavy chain variable region amino acid sequence at least 98% identical to the amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78. The antibody can have a light chain variable region amino acid sequence at least 99% identical to the amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79, and a heavy chain variable region amino acid sequence at least 99% identical to the amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78. The antibody can have a light chain variable region amino as set forth in amino acids 1-97 of SEQ ID NO:79, and a heavy chain variable region amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78.

[009] The antibody can have a light chain variable region amino acid sequence at least 90% identical to the amino acid sequence as set forth in amino acids 1-96 of SEQ ID NO:81, and a heavy chain variable region amino acid sequence at least 90% identical to the amino acid sequence as set forth in amino acids 1-112 of SEQ ID NO:80. The antibody can have a light chain variable region amino acid sequence at least 95% identical to the amino acid sequence as set forth in amino acids 1-96 of SEQ ID NO:81, and a heavy chain variable region amino acid sequence at least 95% identical to the amino acid sequence as set forth in amino acids 1-112 of SEQ ID NO:80. The antibody can have a light chain variable region amino acid sequence at least 98% identical to the amino acid sequence as set forth in amino acids 1-96 of SEQ ID NO:81, and a heavy chain variable region amino acid sequence at least 98% identical to the amino acid sequence as set forth in amino acids 1-112 of SEQ ID NO:80. The antibody can have a light chain variable region amino acid sequence at least 99% identical to the amino acid sequence as set forth in amino acids 1-96 of SEQ ID NO:81, and a heavy chain variable region amino acid sequence at least 99% identical to the amino acid sequence as set forth in amino acids 1-112 of SEQ ID NO:80. The antibody can have a light chain variable region amino as set forth in amino acids 1-96 of SEQ ID NO:81, and a heavy chain variable region amino acid sequence as set forth in amino acids 1-112 of SEQ ID NO:80.

[010] In another aspect, this document features an antibody that binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody is selected from the group consisting of an antibody comprising a light chain amino acid

sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:78; and an antibody comprising a light chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:80.

[011] The antibody can have a light chain amino acid sequence at least 90% identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 90% identical to the amino acid sequence set forth in SEQ ID NO:78. The antibody can have a light chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:79, and a heavy chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:78. The antibody can have a light chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:78. The antibody can have a light chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:78. The antibody can have a light chain amino acid sequence as set forth in SEQ ID NO:79 and a heavy chain amino acid sequence as set forth in SEQ ID NO:78.

[012] The antibody can have a light chain amino acid sequence at least 90% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 90% identical to the amino acid sequence set forth in SEQ ID NO:80. The antibody can have a light chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:80. The antibody can have a light chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:80. The antibody can have a light chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:80. The antibody can have a light chain amino acid sequence as set forth in SEQ ID NO:81 and a heavy chain amino acid sequence as set forth in SEQ ID NO:80.

[013] Any of the antibodies featured herein can be an scFv, Fv, Fab', Fab, diabody, linear antibody or F(ab')2 antigen-binding fragment of an antibody; a CDR, univalent fragment, or a

single domain antibody; a human, humanized or part-human antibody or antigen-binding fragment thereof, or a recombinant antibody. The antibody can be produced in a plant.

[014] Any of the antibodies featured herein can be operatively attached to a biological agent or a diagnostic agent. For example, an antibody can be operatively attached to an agent that cleaves a substantially inactive prodrug to release a substantially active drug. The drug can be an anti-influenza agent. An antibody can be operatively attached to an anti-viral agent (*e.g.*, an anti-influenza agent). An antibody can be operatively attached to a biological agent as a fusion protein prepared by expressing a recombinant vector that comprises, in the same reading frame, a DNA segment encoding the antibody operatively linked to a DNA segment encoding the biological agent. An antibody can be operatively attached to a biological agent via a biologically releasable bond or selectively cleavable linker.

[015] An antibody can be operatively attached to a diagnostic, imaging or detectable agent. For example, an antibody can be operatively attached to an X-ray detectable compound, a radioactive ion or a nuclear magnetic spin-resonance isotope. An antibody can be operatively attached to (a) the X-ray detectable compound bismuth (III), gold (III), lanthanum (III) or lead (II); (b) the detectable radioactive ion copper67, gallium67, gallium68, indium111, indium113, iodine123, iodine125, iodine131, mercury197, mercury203, rhenium186, rhenium188, rubidium97, rubidium103, technetium99m or yttrium90; or (c) the detectable nuclear magnetic spin-resonance isotope cobalt (II), copper (II), chromium (III), dysprosium (III), erbium (III), gadolinium (III), holmium (III), iron (II), iron (III), manganese (II), neodymium (III), nickel (II), samarium (III), terbium (III), vanadium (II) or ytterbium (III). An antibody can be operatively attached to biotin, avidin or to an enzyme that generates a colored product upon contact with a chromogenic substrate.

[016] In another aspect, this document features a nucleic acid comprising a nucleotide sequence encoding an antibody light chain or an antibody heavy chain as provided herein. An expression vector containing the nucleic acid also is provided. The expression vector can further include a nucleotide sequence encoding a leader sequence.

[017] This document also features a host cell containing an expression vector as provided herein. The host cell can be a plant cell.

[018] In addition, this document features a plant comprising a plant cell as provided herein. The plant can be from a genus selected from the group consisting of *Brassica*, *Nicotiana*, *Petunia*, *Lycopersicon*, *Solanum*, *Capsium*, *Daucus*, *Apium*, *Lactuca*, *Sinapis*, or *Arabidopsis*. The plant can be from a species selected from the group consisting of *Nicotiana benthamiana*, *Brassica carinata*, *Brassica juncea*, *Brassica napus*, *Brassica nigra*, *Brassica*

oleraceae, *Brassica tournifortii*, *Sinapis alba*, and *Raphanus sativus*. The plant can be selected from the group consisting of alfalfa, radish, mustard, mung bean, broccoli, watercress, soybean, wheat, sunflower, cabbage, clover, petunia, tomato, potato, tobacco, spinach, and lentil. The plant can be a sprouted seedling.

[019] In another aspect, this document features a recombinant, plant-produced monoclonal antibody that binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody is selected from the group consisting of an antibody comprising a light chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:78; and an antibody comprising a light chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 85 percent identical to the amino acid sequence set forth in SEQ ID NO:80.

[020] The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:79, and a heavy chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:78. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:78. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:79 and a heavy chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:78. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence as set forth in SEQ ID NO:79 and a heavy chain amino acid sequence as set forth in SEQ ID NO:78.

[021] The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 90% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 90% identical to the amino acid sequence set forth in SEQ ID NO:80. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 95% identical to the amino acid sequence set forth in SEQ ID NO:80. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 98% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 98%

identical to the amino acid sequence set forth in SEQ ID NO:80. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:81 and a heavy chain amino acid sequence at least 99% identical to the amino acid sequence set forth in SEQ ID NO:80. The recombinant, plant-produced monoclonal antibody can have a light chain amino acid sequence as set forth in SEQ ID NO:81 and a heavy chain amino acid sequence as set forth in SEQ ID NO:80.

[022] In still another aspect, this document features a pharmaceutical composition comprising an antibody as provided herein, and a pharmaceutically acceptable carrier. The composition can be formulated for parenteral or topical administration. The antibody can be a recombinant, plant-produced antibody. The pharmaceutically acceptable composition can be an encapsulated or liposomal formulation. The composition can further comprise a second therapeutic agent.

[023] This document also features use of a composition as provided herein for treating an influenza infection in a subject in need thereof, as well as use of a composition as provided herein in the manufacture of a medicament for treating an influenza infection.

[024] In another aspect, this document features a method for determining whether a subject is at risk for influenza virus infection. The method can include contacting a biological sample from the subject with an antibody as provided herein. The subject can be a human.

[025] In yet another aspect, this document features a method for typing an influenza virus, comprising contacting the influenza virus with an antibody as provided herein, and if binding of the antibody to the influenza virus is detected, typing the influenza virus as an H5 virus.

[026] This document also features a method for treating a subject in need thereof, comprising contacting a biological sample from the subject with an antibody as provided herein and, if the antibody shows detectable binding to the biological sample, administering an antibody as provided herein to the subject. The subject can be a human. The subject can be diagnosed as having influenza.

[026a] In another aspect, there is provided an isolated monoclonal antibody that specifically binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody comprises the light chain variable region amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79 and the heavy chain variable region amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78.

[026b] In another aspect, there is provided an antibody that specifically binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody comprises the light chain amino acid sequence as set forth in SEQ ID NO:79 and the heavy chain amino acid sequence as set forth in SEQ ID NO:78.

[026c] In another aspect, there is provided a nucleic acid comprising a nucleotide sequence encoding the antibody light chain or antibody heavy chain of the isolated monoclonal antibody as described above.

[026d] In another aspect, there is provided an expression vector comprising the nucleic acid as described herein.

[026e] In another aspect, there is provided a host cell comprising the expression vector as described above.

[026f] In another aspect, there is provided use of a plant comprising the host cell as described above for the production of the antibody light chain or antibody heavy chain of the isolated monoclonal antibody as described above.

[026g] In another aspect, there is provided a recombinant, plant-produced monoclonal antibody that specifically binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody comprises the light chain amino acid sequence as set forth in SEQ ID NO:79 and the heavy chain amino acid sequence as set forth in SEQ ID NO:78.

[026h] In another aspect, there is provided a pharmaceutical composition comprising the antibody as described above, and a pharmaceutically acceptable carrier.

[026i] In another aspect, there is provided use of the pharmaceutical composition as described above for treating an influenza infection in a subject in need thereof, wherein the influenza infection is caused by an H5 influenza virus.

[026j] In another aspect, there is provided use of the pharmaceutical composition as described above in the manufacture of a medicament for treating an influenza infection, wherein the influenza infection is caused by an H5 influenza virus.

[026k] In another aspect, there is provided use of the antibody as described above for determining whether a subject is at risk for influenza virus infection, wherein the influenza infection is caused by an H5 influenza virus.

[026l] In another aspect, there is provided a method for typing an influenza virus, comprising contacting the influenza virus with the antibody of as described herein, and if binding of the antibody to the influenza virus is detected, typing the influenza virus as an H5 virus.

[026m] In another aspect, there is provided use of the antibody as described above for treating influenza in a subject in need thereof, wherein the influenza infection is caused by an H5 influenza virus, and wherein the subject has been selected for treatment by a process comprising contacting a biological sample from the subject with the antibody as described above and, if the antibody shows detectable binding to the biological sample, selecting the subject for treatment with the antibody.

[027] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. In case of conflict, the present specification,

including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

[028] The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[029] Figure 1 depicts the plant viral vector pGRD4-H5 HA.

[030] Figure 2A is a graph depicting the activity of mAb 4F5 against homologous and heterologous strains of influenza viruses. Figure 2B is a graph depicting the activity of mAb 5F5 against homologous and heterologous strains of influenza viruses. Figure 2C is a graph depicting the activity of mAb 1E11 against homologous and heterologous strains of influenza viruses.

[031] Figure 3 is a table summarizing hemagglutinin inhibition activity of anti-HA mAbs.

[032] Figure 4 is a table summarizing binding activity of anti-HA mAbs.

[033] Figure 5 is a table summarizing hemagglutination inhibition activity of anti-H5 HA mAbs.

[034] Figure 6 is a table summarizing hemagglutination inhibition activity of anti-H3 HA mAbs.

[035] Figure 7 depicts the experimental design used to evaluate the protective efficacy of mAbs in mice.

[036] Figure 8 depicts the results of an experiment to evaluate the protective efficacy of mAbs in mice.

[037] Figure 9 depicts the amino acid sequences, including signal peptide sequences, of heavy and light chains for mAbs 1E11 and 4F5.

[038] Figure 10 depicts the amino acid sequences, without signal peptide sequences, of heavy and light chains for mAbs 1E11 and 4F5.

DETAILED DESCRIPTION

[039] This document relates to influenza antibodies that can be useful to prevent, delay onset of, treat, ameliorate symptoms of, reduce occurrence of, and/or diagnose influenza infection. This document also relates to antibody compositions, and methods of production

of provided antibody compositions, including but not limited to, production in plant systems. Further, this document relates to vectors, fusion proteins, plant cells, plants and compositions comprising antibodies or antigen binding fragments thereof. Still further provided are kits as well as therapeutic and diagnostic uses in association with influenza infection in a subject.

Influenza Antigens

[040] In general, influenza antigens can include any immunogenic polypeptide that elicits an immune response against influenza virus. Immunogenic polypeptides of interest can be provided as independent polypeptides, as fusion proteins, as modified polypeptides [*e.g.*, containing additional pendant groups such as carbohydrate groups, alkyl groups (such as methyl groups, ethyl groups, or propyl groups), phosphate groups, lipid groups, amide groups, formyl groups, biotinyl groups, heme groups, hydroxyl groups, iodo groups, isoprenyl groups, myristoyl groups, flavin groups, palmitoyl groups, sulfate groups, or polyethylene glycol]. In some embodiments, influenza antigen polypeptides for use in accordance with this disclosure have an amino acid sequence that is or includes a sequence identical to that of an influenza polypeptide found in nature; in some embodiments influenza antigen polypeptides have an amino acid sequence that is or includes a sequence identical to a characteristic portion (*e.g.*, an immunogenic portion) of an influenza polypeptide found in nature.

[041] In certain embodiments, full length proteins are utilized as influenza antigen polypeptides in vaccine compositions in accordance with this disclosure. In some embodiments, one or more immunogenic portions of influenza polypeptides are used. In certain embodiments, two or three or more immunogenic portions are utilized, as one or more separate polypeptides or linked together in one or more fusion polypeptides.

[042] Influenza antigen polypeptides can include, for example, full-length influenza polypeptides, fusions thereof, and/or immunogenic portions thereof. Where portions of influenza proteins are utilized, whether alone or in fusion proteins, such portions retain immunological activity (*e.g.*, cross-reactivity with anti-influenza antibodies). Based on their capacity to induce immunoprotective response against viral infection, hemagglutinin is an antigen of interest in generating vaccines.

[043] In certain embodiments, full length hemagglutinin (HA) is utilized to generate HA antibodies as provided herein. In some embodiments one or more domains of HA can be used. In certain embodiments, two or three or more domains are utilized, as one or more

separate polypeptides or linked together in one or more fusion polypeptides. Sequences of exemplary HA polypeptides are presented in Table 1.

Table 1: Exemplary HA Sequences

GenBank Accession	**Strain**	**HA Sequence**
ABY51347	A/environment /New York/3181-1/2006 (H7N2)	5'MNIQILAFIACVLTGAKGDKICLGHHAVANGTKVNTLTEKGI EVVNATETVETADVKKICTQGKRATDLGRCGLLGTLIGPPQCD QFLEFSSDLIIERREGTDVCYPGRFTNEESLRQILRRSGGIGKES MGFTYSGIRTNGAASACTRSGSSFYAEMKWLLSNSDNSAFPQ MTKAYRNPRNKPALIIWGVHHSESASEQTKLYGSGNKLITVRS SKYQQSFTPSPGTRRIDFHWLLLDPNDTVTFTFNGAFIAPDRAS FFRGESLGVQSDAPLDSSCRGDCFHSGGTIVSSLPFQNINSRTV GRCPRYVKQKSLLLATGMRNVPEKPKPRGLFGAIAGFIENGW EGLINGWYGFRHQNAQGEGTAADYKSTQSAIDQITGKLNRLIG KTNQQFELIDNEFNEIEQQIGNVINWTRDAMTEIWSYNAELLV AMENQHTIDLADSEMSKLYERVKKQLRENAEEDGTGCFEIFH KCDDQCMESIRNNTYDHTQYRTESLQNRIQIDPVKLSSGYKDII LWFSFGASCFILLAIAMGLVFICIKNGNMQCTICI 3' (SEQ ID NO:1)
ACC61810	A/environment /New York/3185-1/2006 (H7N2)	5'MNTQILAFIACVLTGVKGDKICLGHHAVANGTKVNTLTEKG IEVVNATETVETADVKKICTQGKRATDLGRCGLLGTLIGPPQC DQFLEFSSDLIIERREGTDVCYPGRFTNEESLRQILRRSGGIGKE SMGFTYSGIRTNGATSACTRSGSSFYAEMKWLLSNSDNSAFPQ MTKAYRNPRNKPALIIWGVHHSESVSEQTKLYGSGNKLITVRS SKYQQSFTPSPGARRIDFHWLLLDPNDTVTFTFNGAFIAPDRAS FFRGESLGVQSDVPLDSSCRGDCFHSGGTIVSSLPFQNINSRTV GKCPRYVKQKSLLLATGMRNVPEKPKPRGLFGAIAGFIENGW EGLINGWYGFRHQNAQGEGTAADYKSTQSAIDQITGKLNRLIG KTNQQFELIDNEFNEIEQQIGNVINWTRDAMTEIWSYNAELLV AMENQHTIDLADSEMSKLYERVKKQLRENAEEDGTGCFEIFH KCDDQCMESIRNNTYDHTQYRTESLQNRIQIDPVKLSSGYKDII LWFSFGASCFLLLAIAMGLVFICIKNGNMQCTICI 3' (SEQ ID NO:2)

ABI26075	A/guineafowl/NY/4649-18/2006 (H7N2)	5'MNIQILAFIACVLTGAKGDKICLGHHAVANGTKVNTLTEKGI EVVNATETVETANIKKICTQGKRPTDLGQCGLLGTLIGPPQCD QFLEFSSDLIIERREGTDVCYPGKFTNEESLRQILRRSGGIGKES MGFTYSGIRTNGATSACTRSGSSFYAEMKWLLSNSDNAAFPQ MTKSYRNPRNKPALIIWGVHHSESVSEQTKLYGSGNKLIKVRS SKYQQSFTPNPGARRIDFHWLLLDPNDTVTFTFNGAFIAPDRA SFFRGESIGVQSDAPLDSSCGGNCFHNGGTIVSSLPFQNINPRTV GKCPRYVKQKSLLLATGMRNVPEKPKKRGLFGAIAGFIENGW EGLINGWYGFRHQNAQGEGTAADYKSTQSAIDQITGKLNRLIG KTNQQFELINNEFNEVEQQIGNVINWTQDAMTEVWSYNAELL VAMENQHTIDLTDSEMSKLYERVRKQLRENAEEDGTGCFEIF HKCDDHCMESIRNNTYDHTQYRTESLQNRIQIDPVKLSGGYK DIILWFSFGASCFLLLAIAMGLVFICIKNGNMQCTICI 3' (SEQ ID NO:3)
ABR37506	A/environment/Maryland/267/2006 (H7N3)	5'MNTQILALIAYMLIGAKGDKICLGHHAVANGTKVNTLTERG IEVVNATETVETVNIKKICTQGKRPTDLGQCGLLGTLIGPPQCD QFLEFDADLIIERREGTDVCYPGKFTNEESLRQILRGSGGIDKES MGFTYSGIRTNGVTSACRRSGSSFYAEMKWLLSNSDNAAFPQ MTKSYRNPRNKPALIIWGVHHSGSATEQTKLYGSGNKLITVGS SKYQQSFTPSPGARPQVNGQSGRIDFHWLLLDPNDTVTFTFNG AFIAPDRASFFRGESLGVQSDVPLDSGCEGDCFHSRGTIVSSLP FQNINPRTVGKCPRYVKQTSLLLATGMRNVPENPKTRGLFGAI AGFIENGWEGLIDGWYGFRHQNAQGEGTAADYKSTQSAIDQI TGKLNRLIDKTNQQFELIDNEFSEIEQQIGNVINWTRDSMTEV WSYNAELLVAMENQHTIDLADSEMNKLYERVRKQLRENAEE DGTGCFEIFHKCDDQCMESIRNNTYDHTQYRTESLQNRIQIDP VKLSSGYKDIILWFSFGASCFLLLAIAMGLVFICIKNGNMRCTI CI 3' (SEQ ID NO:4)
ACF47475	A/mallard/California/HKWF1971/2007 (H7N7)	5'MNTQILALIACMLIGAKGDKICLGHHAVANGTKVNTLTERG IEVVNATETVETANIKKICTQGKRPTDLGQCGLLGTLIGPPQCD QFLEFDADLIIERREGTDVCYPGKFTNEESLRQILRGSGGIDKES MGFTYSGIRTNGATSACRRSGSSFYAEMKWLLSNSDNAAFPQ MTKSYRNPRNKPALIIWGVHHSGSATEQTKLYGSGNKLITVGS SKYQQSFTPSPGARPQVNGQSGRIDFHWLLLDPNDTVTFTFNG AFIAPDRASFFRGGSLGVQSDVPLDSGCEGDCFHSGGTIVSSLP FQNINPRTVGKCPRYVKQTSLLLATGMRNVPENPKTRGLFGAI AGFIENGWEGLIDGWYGFRHQNAQGEGTAADYKSTQSAIDQI TGKLNRLIDKTNQQFELIDNEFNEIEQQIGNVINWTRDSMTEV WSYNAELLVAMENQHTIDLADSEMNKLYERVRKQLRENAEE DGTGCFEIFHKCDDQCMESIRNNTYDHTQYRTESLQNRIQINP VKLSSGYKDIILWFSFGASCFLLLAIAMGLVFICIKNGNMRCTI CI 3' (SEQ ID NO:5)

ABP96852	A/Egypt/2616-NAMRU3/2007 (H5N1)	5'MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFLNVPEWSYIVEKINPANDLCYPGDFNDYEELKHLLSRINHFE KIQIIPKSSWSDYEASSGVSSACPYQGRSSFFRNVVWLIKKNNA YPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQIRLYQNPTTYISI GTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKSNDAINFES NGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPIGAINS SMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQGERRRRKR GLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKEST QKAIDGVTNKVNSIINKMNTQFEAVGREFNNLERRIENLNKK MEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRL QLRDNAKELGNGCFEFYHRCDNECMESVRNGTYDYPQYSEE ARLKREEISGVKLESMGIYQILSIYSTVASSLALAIMVAGLFLW MCSNGSLQCRICI 3' (SEQ ID NO:6)
ABV23934	A/Nigeria/6e/07 (H5N1)	5'DQICIGYHANNSTEQVDTIMEKNVTVTHAQNILEKTHNGKL CDLDGVKPLILRDCSVAGWLLGNPMCDEFLNVPEWSYIVEKI NPANDLCYPGNFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEA SSGVSSACPYQGRSSFFRNVVWLIKKDNAYPTIKRSYNNTNQE DLLVLWGIHHPNDAAEQTRLYQNPTTYISVGTSTLNQRLVPKI ATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPENAYKI VKKGDSTIMKSELEYGNCNTKCQTPIGAINSSMPFHNIHPLTIG ECPKYVKSNKLVLATGLRNSPQGERRRKKRGLFGAIAGFIEGG WQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVN SIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYN AELLVLMENERTLDFHDSNVKNLYDKIRLQLRDNAKELGNGC FEFYHRCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLES IGTYQILSIYSTVASSLTLAIMVAGLSLWMCSNGSLQCRICI 3' (SEQ ID NO:7)
ABI16504	A/China/GD01/2006 (H5N1)	5'MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFINVPEWSYIVEKANPANDLCYPGNFNDYEELKHLLSRINHF EKIQIISKSSWSDHEASSGVSSACPYQGTPSFFRNVVWLIKKNN TYPTIKRSYNNTNQEDLLILWGIHHSNNAAEQTKLYQNPTTYIS VGTSTLNLRLVPKIATRSKVNGQSGRMDFFWTILKPNDAINFE SNGNFIAPEYAYKIVKKGDSAIMKSEVEYGNCNTKCQTPIGAI NSSMPFHNIHPLTIGECPKYVKSNKLVLATGLRNSPLRERRRK RGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKES TQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKK MEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRL QLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEE ARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMVAGLSLW MCSNGSLQCRICI 3' (SEQ ID NO:8)

ABY27653	A/India/m777/2007 (H5N1)	5'MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKKHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHF EKIQIIPKSSWSSHEASLGVSSACPYQGKTSFFRNVVWLIKKNS TYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYI SVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTILKPNDAINF ESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRETRGL FGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQK AIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMED GFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLR DNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEEARL KREEISGVKLESIGIYQILSIYSTVASSLALAIMVAGLSLWMCSN GSLQCRIC 3' (SEQ ID NO:9)
ABI36046	A/Indonesia/CDC326N/2006 (H5N1)	5'DQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKL CDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKA NPTNDLCYPGSFNDYEELKHLLSRINHFEKIQIIPKSSWSDHEAS SGVSSACPYLGSPSFFRNVVWLIKKNSTYPTIKKSYNNTNQED LLVLWGIHHPNDAAEQTRLYQNPTTYISIGTSTLNQRLVPKIAT RSKVNGQSGRMEFFWTILNPNDAINFESNGNFIAPEYAYKIVK KGDSAIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGE CPKYVKSNRLVLATGLRNSPQRESRRKKRGLFGAIAGFIEGGW QGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSI IDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAE LLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCF EFYHKCDNECMESIRNGTYNYPQYSEEARLKREEISGVKLESI GTYQILSIYSTVASSLALAIMMAGLSLWMCSNGSLQCRICI 3' (SEQ ID NO:10)
ACD85624	A/Mississippi/05/2008 (H3N2)	5'MKTIIALSYILCLVSAQKFPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGEICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLV ASSGTLEFNNESFNWTGVTQNGTSSACIRRSNNSFFSRLNWLT HLKFKYPALNVTMPNNEEFDKLYIWGVHHPGTDNDQIFLYAQ ASGRITVSTKRSQQTVIPNIGSRPRVRNIPSRISIYWTIVKPGDIL LINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSIP NDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGI FGAIAGFIENGWEGMVDGWYGFRHQNSEGIGQAADLKSTQA AIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT KIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTKKQLREN AEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRF QIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN IRCNICI 3' (SEQ ID NO:11)

ACF10321	A/New York/06/2008 (H3N2)	5'MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGEICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLV ASSGTLEFKNESFNWTGVTQNGTSSACIRRSNNSFFSRLNWLT HLKFKYPALNVTMPNKEKFDKLYIWGVHHPGTDNDQIFLYAQ ASGRITVSTKRSQQTVIPNIGSRLRVRDIPSRISIYWTIVKPGDIL LINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSIP NDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGI FGAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQA AIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT KIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTKKQLREN AEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRF QIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN IRCNICI 3' (SEQ ID NO:12)
ACD85628	A/Idaho/03/2008 (H3N2)	5'MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGEICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSKCYPYDVPDYASLRSLV ASSGTLEFNNESFNWTGVTQNGTSSACIRRSNNSFFSRLNWLT HLKFKYPALNVTMPNNEKFDKLYIWGVHHPGTDNDQIFLYAQ ASGRITVSTKRSQQTVIPNIGSRPRVRDIPSRISIYWTIVKPGDIL LINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSIP NDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGI FGAIAGFIENGWEGMVDGWYGFRHQNSEGIGQAADLKSTQA AIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT KIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTKKQLREN AEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRF QIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN IRCNICI 3' (SEQ ID NO:13)
ACF40065	A/Louisiana/06/2008 (H3N2)	5'MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGEICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLV ASSGTLEFNNESFNWTGVTQNGTSSACIRRSNNSFFSRLNWLT HLKFKYPALNVTMPNNEKFDKLYIWGVHHPGTDNDQIFLYAQ ASGRITVSTKRSQQTVIPNIGSRPRVRNIPSRISIYWTIVKPGDIL LINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSIP NDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGI FGAIAGFIENGWEGMVDGWYGFRHQNSEGIGQAADLKSTQA AIDQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT KIDLWSYNAELLVALENQHTIDLTDSEMNKLFEKTKKQLREN AEDMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRF QIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN IRCNICI 3' (SEQ ID NO:14)

ACB11768	A/Indiana/01/2008 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNV TVTHSVNLLENSHNGKLCLLKGIAPLQLGNCSVAGWILGNPEC ELLISKESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSF ERFEIFPKESSWPNHTVTGVSASCSHNGESSFYRNLLWLTGKN GLYPNLSKSYANNKEKEVLVLWGVHHPPNIGDQKALYHTEN AYVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYHWTLLEPGDT IIFEANGNLIAPRYAFTLSRGFGSGIINSNAPMDKCDAKCQTPQ GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSR GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKST QNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKK VDDGFIDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESK LNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWM CSNGSLQCRICI 3' (SEQ ID NO:15)
ACB11769	A/Pennsylvania/02/2008 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNV TVTHSVNLLENSHNGKLCLLKGIAPLQLGNCSVAGWILGNPEC ELLISKESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSF ERFEIFPKESSWPNHTVTGVSASCSHNGESSFYRNLLWLTGKN GLYPNLSKSYANNKEKEVLVLWGVHHPPNIGDQKTLYHTENA YVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYYWTLLEPGDTII FEANGNLIAPRYAFALSRGFGSGIINSNAPMDKCDAKCQTPQG AINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRG LFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKSTQ NAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKV DDGFIDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQL KNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKL NREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMC SNGSLQCRICI 3' (SEQ ID NO:16)
ACD47238	A/Alaska/02/2008 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNV TVTHSVNLLENSHNGKLCLLKGIAPLQLGNCSVAGWILGNPEC ELLISKESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSF ERFEIFPKESAWPNHTVTGVSASCSHNGEXSFYRNLLWLTXKN GLYPNLSKSYANNKEKEVLVLWGVHHPPNIGDQKALYHTEN AYVSVVSSHYSRKFTPEIAKRPKVRXQEGRINYYWTLLEPGDT IIFEANGNLIAPRYAFALSRGFGSGIINSNAPMDKCDAKCQTPQ GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSR GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKST QNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKK VDDGFIDIWTYNAELLVLLENERTLDFHDSNXKNLYEKVKSQ LKNNAKEIGNGCFEFYHKCNDECMESVKNGTXDYPKYSEESK LNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWM CSNGSLQCRICI 3' (SEQ ID NO:17)

ACD85766	A/Indiana/04/2008 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLENNHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSFERFEMFPKEGSWPNHTVTGVSASCSHNGESSFYRNLLWLTGKNGLYPNLXKSYANNKEKEVLVLWGVHHPPNIGDQKALYHTENAYVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPRYAFALSRGFGSGIINSNAPMDNCDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVXKEFNKLERRMENLNKKVDDGFIDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI 3' (SEQ ID NO:18)
ACF40125	A/Wisconsin/01/2008 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLENSHNGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGESSFYRNLLWLTGKNGLYPNLSKSYANNKEKEVLVLWGVHHPPDIGDQKTLYHTENAYVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPRYAFALSRGFGSGIINSNAPMDKCDAKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFIDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI 3' (SEQ ID NO:19)
	Vietnam H5N1	5'AKAGVQSVKMEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTHAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHFEKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNSTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYISVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGIYQILSIYSTVASSLALALMVAGLSLWMCSNGSLQCRICI 3' (SEQ ID NO:20)

	Wyoming H3N2	5'MKTIIALSYILCLVFSQKLPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGGICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLV ASSGTLEFNNESFNWAGVTQNGTSSACKRRSNKSFFSRLNWL THLKYKYPALNVTMPNNEKFDKLYIWGVHHPVTDSDQISLYA QASGRITVSTKRSQQTVIPNIGYRPRVRDISSRISIYWTIVKPGDI LLINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSI PNDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRG IFGAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQA AINQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT KIDLWSYNAELLVALENQHTIDLTDSEMNKLFERTKKQLREN AEDMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEALNNRF QIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN IRCNICI 3' (SEQ ID NO:21)
DQ371928	A/Anhui/1/2005 (H5N1)	5'MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFINVPEWSYIVEKANPANDLCYPGNFNDYEELKHLLSRINHF EKIQIIPKSSWSDHEASSGVSSACPYQGTPSFFRNVVWLIKKNN TYPTIKRSYNNTNQEDLLILWGIHHSNDAAEQTKLYQNPTTYIS VGTSTLNQRLVPKIATRSKVNGQSGRMDFFWTILKPNDAINFE SNGNFIAPEYAYKIVKKGDSAIVKSEVEYGNCNTKCQTPIGAIN SSMPFHNIHPLTIGECPKYVKSNKLVLATGLRNSPLRERRRKR GLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKEST QKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKK MEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRL QLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQYSEE ARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMVAGLSLW MCSNGSLQCRICI 3' (SEQ ID NO:22)
ISDN125873	A/Indonesia/5/05	5'MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFINVPEWSYIVEKANPTNDLCYPGSFNDYEELKHLLSRINHFE KIQIIPKSSWSDHEASSGVSSACPYLGSPSFFRNVVWLIKKNST YPTIKKSYNNTNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYIS IGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFES NGNFIAPEYAYKIVKKGDSAIMKSELEYGNCNTKCQTPMGAIN SSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRESRRKKR GLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADKEST QKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKK MEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRL QLRDNAKELGNGCFEFYHKCDNECMESIRNGTYNYPQYSEEA RLKREEISGVKLESIGTYQILSIYSTVASSLALAIMMAGLSLWM CSNGSLQCRICI 3' (SEQ ID NO:23)

DQ137873	A/bar-headed goose/Qinghai /0510/05 (H5N1)	5'MERIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFLNVPEWSYIVEKINPANDLCYPGNFNDYEELKHLLSRINHFE RIQIIPKSSWSDHEASSGVSSACPYQGRSSFFRNVVWLIKKNNA YPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTRLYQNPTTYIS VGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFE SNGNFIAPENAYKNCQKGDSTIMKSELEYGNCNTKCQTPIGAI NSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQGERRRK KRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADK ESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLN KKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDK VRLQLRDNAKELGNGCFEFYHRCDNECMESVRNGTYDYPQY SEEARLKREEISGVKLESIGTYQILSIYSTVASSLALAIMVAGLS LWMCSNG 3' (SEQ ID NO:24)
	A/VietNam/1194/04	5'MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTV THAQDILEKTHNGKLCDLDGVKPLILRDCSVAGWLLGNPMCD EFINVPEWSYIVEKANPVNDLCYPGDFNDYEELKHLLSRINHF EKIQIIPKSSWSSHEASLGVSSACPYQGKSSFFRNVVWLIKKNS TYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYI SVGTSTLNQRLVPRIATRSKVNGQSGRMEFFWTILKPNDAINF ESNGNFIAPEYAYKIVKKGDSTIMKSELEYGNCNTKCQTPMGA INSSMPFHNIHPLTIGECPKYVKSNRLVLATGLRNSPQRERRRK KRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQGSGYAADK ESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLN KKMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDK VRLQLRDNAKELGNGCFEFYHKCDNECMESVRNGTYDYPQY SEEARLKREEISGVKLESIGIYQILSIYSTVASSLALAIMVAGLSL WMCSNGSLQCRICI 3' (SEQ ID NO:25)
	B/Brisbane/3/07	5'MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVT GVIPLTTTPTKSYFANLKGTKTRGKLCPDCLNCTDLDVALGRP MCVGTTPSAKASILHEVRPVTSGCFPIMHDRTKIRQLANLLRG YENIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKSGFFATM AWAVPKDNNKNATNPLTVEVPYICTEGEDQITVWGFHSDDKT QMKNLYGDSNPQKFTSSANGVTTHYVSQIGGFPDQTEDGGLP QSGRIVVDYMMQKPGKTGTIVYQRGVLLPQKVWCASGRSKVI KGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWV KTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAG WHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKN LQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEG IINSEDEHLLALERKLKKMLGPSAVDIGNGCFETKHKCNQTCL DRIAAGTFNAGEFSLPTFDSLNITAASLNDDGLDNHTILLYYST AASSLAVTLMLAIFIVYMVSRDNVSCSICL 3' (SEQ ID NO:26)

ACA28844	A/Brisbane/59/2007 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNV TVTHSVNLLENSHNGKLCLLKGIAPLQLGNCSVAGWILGNPEC ELLISKESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSF ERFEIFPKESSWPNHTVTGVSASCSHNGESSFYRNLLWLTGKN GLYPNLSKSYANNKEKEVLVLWGVHHPPNIGDQKALYHTEN AYVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYYWTLLEPGDT IIFEANGNLIAPRYAFALSRGFGSGIINSNAPMDKCDAKCQTPQ GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSR GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKST QNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKK VDDGFIDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESK LNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWM CSNGSLQCRICI 3' (SEQ ID NO:27)
	A/Brisbane/10/2007 (H3N2)	5'QKLPGNDNSTATLCLGHHAVPNGTIVKTITNDQIEVTNATEL VQSSSTGEICDSPHQILDGENCTLIDALLGDPQCDGFQNKKWD LFVERSKAYSNCYPYDVPDYASLRSLVASSGTLEFNNESFNWT GVTQNGTSSACIRRSNNSFFSRLNWLTHLKFKYPALNVTMPN NEKFDKLYIWGVHHPGTDNDQIFPYAQASGRITVSTKRSQQTV IPNIGSRPRVRNIPSRISIYWTIVKPGDILLINSTGNLIAPRGYFKI RSGKSSIMRSDAPIGKCNSECITPNGSIPNDKPFQNVNRITYGAC PRYVKQNTLKLATGMRNVPEKQTRGIFGAIAGFIENGWEGMV DGWYGFRHQNSEGIGQAADLKSTQAAIDQINGKLNRLIGKTN EKFHQIEKEFSEVEGRIQDLEKYVEDTKIDLWSYNAELLVALE NQHTIDLTDSEMNKLFEKTKKQLRENAEDMGNGCFKIYHKCD NACIGSIRNGTYDHDVYRDEALNNRFQIKGVELKSGYKDWIL WISFAISCFLLCVALLGFIMWACQKGNIRCNI 3' (SEQ ID NO:28)
ACA33493	B/Florida/4/2006	5'MKAIIVLLMVVTSNADRICTGITSSNSPHVVKTATQGEVNVT GVIPLTTTPTKSYFANLKGTRTRGKLCPDCLNCTDLDVALGRP MCVGTTPSAKASILHEVKPVTSGCFPIMHDRTKIRQLPNLLRG YENIRLSTQNVIDAEKAPGGPYRLGTSGSCPNATSKSGFFATM AWAVPKDNNKNATNPLTVEVPYICTEGEDQITVWGFHSDDKT QMKNLYGDSNPQKFTSSANGVTTHYVSQIGSFPDQTEDGGLP QSGRIVVDYMMQKPGKTGTIVYQRGVLLPQKVWCASGRSKVI KGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWV KTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAG WHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKN LQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEG IINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCL DRIAAGTFNAGEFSLPTFDSLNITAASLNDDGLDNHTILLYYST AASSLAVTLMLAIFIVYMVSRDNVSCSICL 3' (SEQ ID NO:29)

	B/Malaysia/2506/2004-like	5'MKAIIVLLMVVTSNADRIICTGITSSNSPHVVKTATQGEVNV TGVIPLTTTPTKSHFANLKGTETRGKLCPKCLNCTDLDVALGR PKCTGNIPSARVSILHEVRPVTSGCFPIMHDRTKIRQLPNLLRG YEHIRLSTHNVINAENAPGGPYKIGTSGSCPNVTNGNGFFATM AWAVPKNDNNKTATNSLTIEVPYICTEGEDQITVWGFHSDNET QMAKLYGDSKPQKFTSSANGVTTHYVSQIGGFPNQTEDGGLP QSGRIVVDYMVQKSGKTGTITYQRGILLPQKVWCASGRSKVI KGSLPLIGEADCLHEKYGGLNKSKPYYTGEHAKAIGNCPIWV KTPLKLANGTKYRPPAKLLKERGFFGAIAGFLEGGWEGMIAG WHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSLSELEVKN LQRLSGAMDELHNEILELDEKVDDLRADTISSQIELAVLLSNEG IINSEDEHLLALERKLKKMLGPSAVEIGNGCFETKHKCNQTCL DRIAAGTFDAGEFSLPTFDSLNITAASLNDDGLDNHTILLYYST AASSLAVTLMIAIFVVYMVSRDNVSCSICL 3' (SEQ ID NO:30)
AAP34324	A/New Caledonia/20/99 (H1N1)	5'MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNV TVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPEC ELLISKESWSYIVETPNPENGTCYPGYFADYEELREQLSSVSSF ERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKN GLYPNLSKSYVNNKEKEVLVLWGVHHPPNIGNQRALYHTEN AYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDT IIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDECDAKCQTPQ GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSR GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKST QNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKK VDDGFLDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEIGNGCFEFYHKCNNECMESVKNGTYDYPKYSEESK LNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWM CSNGSLQCRICI 3' (SEQ ID NO:31)
ABU99109	A/Solomon Islands/3/2006 (H1N1)	5'MKVKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNV TVTHSVNLLEDSHNGKLCLLKGIAPLQLGNCSVAGWILGNPEC ELLISRESWSYIVEKPNPENGTCYPGHFADYEELREQLSSVSSF ERFEIFPKESSWPNHTTTGVSASCSHNGESSFYKNLLWLTGKN GLYPNLSKSYANNKEKEVLVLWGVHHPPNIGDQRALYHKEN AYVSVVSSHYSRKFTPEIAKRPKVRDQEGRINYYWTLLEPGDT IIFEANGNLIAPRYAFALSRGFGSGIINSNAPMDECDAKCQTPQ GAINSSLPFQNVHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSR GLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSGYAADQKST QNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKK VDDGFIDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESK LNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWM CSNGSLQCRICI 3' (SEQ ID NO:32)

	A/Wisconsin/67/2005 (H3N2)	5'MKTIIALSYILCLVFAQKLPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGGICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLV ASSGTLEFNDESFNWTGVTQNGTSSSCKRRSNNSFFSRLNWLT HLKFKYPALNVTMPNNEKFDKLYIWGVHHPVTDNDQIFLYAQ ASGRITVSTKRSQQTVIPNIGSRPRIRNIPSRISIYWTIVKPGDILL INSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSIPN DKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRGIF GAIAGFIENGWEGMVDGWYGFRHQNSEGIGQAADLKSTQAAI NQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDTKI DLWSYNAELLVALENQHTIDLTDSEMNKLFERTKKQLRENAE DMGNGCFKIYHKCDNACIGSIRNGTYDHDVYRDEALNNRFQI KGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGNIR CNICI 3' (SEQ ID NO:33)
AAT08000	A/Wyoming/3/03 (H3N2)	5'MKTIIALSYILCLVFSQKLPGNDNSTATLCLGHHAVPNGTIV KTITNDQIEVTNATELVQSSSTGGICDSPHQILDGENCTLIDALL GDPQCDGFQNKKWDLFVERSKAYSNCYPYDVPDYASLRSLV ASSGTLEFNNESFNWAGVTQNGTSSACKRRSNKSFFSRLNWL THLKYKYPALNVTMPNNEKFDKLYIWGVHHPVTDSDQISLYA QASGRITVSTKRSQQTVIPNIGYRPRVRDISSRISIYWTIVKPGDI LLINSTGNLIAPRGYFKIRSGKSSIMRSDAPIGKCNSECITPNGSI PNDKPFQNVNRITYGACPRYVKQNTLKLATGMRNVPEKQTRG IFGAIAGFIENGWEGMVDGWYGFRHQNSEGTGQAADLKSTQA AINQINGKLNRLIGKTNEKFHQIEKEFSEVEGRIQDLEKYVEDT KIDLWSYNAELLVALENQHTIDLTDSEMNKLFERTKKQLREN AEDMGNGCFKIYHKCDNACIESIRNGTYDHDVYRDEALNNRF QIKGVELKSGYKDWILWISFAISCFLLCVALLGFIMWACQKGN IRCNICI 3' (SEQ ID NO:34)
AAR02640	A/Netherlands/219/03 (H7N7)	5'SKSRGYKMNTQILVFALVASIPTNADKICLGHHAVSNGTKV NTLTERGVEVVNATETVERTNVPRICSKGKRTVDLGQCGLLG TITGPPQCDQFLEFSADLIIERREGSDVCYPGKFVNEEALRQILR ESGGIDKETMGFTYSGIRTNGTTSACRRSGSSFYAEMKWLLSN TDNAAFPQMTKSYKNTRKDPALIIWGIHHSGSTTEQTKLYGSG NKLITVGSSNYQQSFVPSPGARPQVNGQSGRIDFHWLILNPND TVTFSFNGAFIAPDRASFLRGKSMGIQSEVQVDANCEGDCYHS GGTIISNLPFQNINSRAVGKCPRYVKQESLLLATGMKNVPEIPK RRRRGLFGAIAGFIENGWEGLIDGWYGFRHQNAQGEGTAADY KSTQSAIDQITGKLNRLIEKTNQQFELIDNEFTEVERQIGNVIN WTRDSMTEVWSYNAELLVAMENQHTIDLADSEMNKLYERVK RQLRENAEEDGTGCFEIFHKCDDDCMASIRNNTYDHSKYREE AIQNRIQIDPVKLSSGYKDVILWFSFGASCFILLAIAMGLVFICV KNGNMRCTICI 3' (SEQ ID NO:35)

[044] While sequences of exemplary influenza antigen polypeptides are provided herein, it will be appreciated that any sequence having immunogenic characteristics of HA may be employed. In some embodiments, an influenza antigen polypeptide can have an amino acid sequence that is about 60% identical, about 70% identical, about 80% identical, about 85%

identical, about 90% identical, about 91% identical, about 92% identical, about 93% identical, about 94% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical, about 99% identical, or 100% identical to a sequence selected from the group consisting of SEQ ID NOS:1-35. In some embodiments, such an influenza antigen polypeptide retains immunogenic activity.

[045] In some embodiments, an influenza antigen polypeptide can have an amino acid sequence that comprises about 100 contiguous amino acids of a sequence selected from the group consisting of SEQ ID NOS:1-35. In some embodiments, an influenza antigen polypeptide has an amino acid sequence which is about 60% identical, about 70% identical, about 80% identical, about 85% identical, about 90% identical, about 91% identical, about 92% identical, about 93% identical, about 94% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical, about 99% identical, or 100% identical to a contiguous stretch of about 100 amino acids of a sequence selected from the group consisting of SEQ ID NOS:1-35.

[046] In some embodiments, an influenza antigen polypeptide can have an amino acid sequence that comprises about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, or more contiguous amino acids of a sequence selected from the group consisting of SEQ ID NOS:1-35. In some embodiments, an influenza antigen polypeptide has an amino acid sequence which is about 60% identical, about 70% identical, about 80% identical, about 85% identical, about 90% identical, about 91% identical, about 92% identical, about 93% identical, about 94% identical, about 95% identical, about 96% identical, about 97% identical, about 98% identical, about 99% identical, or 100% identical to a contiguous stretch of about 150, 200, 250, 300, 350, or more amino acids of a sequence selected from the group consisting of SEQ ID NOS:1-35.

[047] For example, sequences having sufficient identity to influenza antigen polypeptide(s) which retain immunogenic characteristics are capable of binding with antibodies which react with one or more antigens provided herein. Immunogenic characteristics often include three dimensional presentation of relevant amino acids or side groups. One skilled in the art can readily identify sequences with modest differences in sequence (*e.g.*, with difference in boundaries and/or some sequence alternatives, that, nonetheless preserve immunogenic characteristics).

[048] In some embodiments, particular portions and/or domains of any of the exemplary sequences set forth in SEQ ID NOS:1-35 may be omitted from an influenza polypeptide. For example, HA polypeptides typically contain a transmembrane anchor sequence. HA

polypeptides in which the transmembrane anchor sequence has been omitted are contemplated herein.

[049] As exemplary antigens, we have utilized sequences from hemagglutinin of particular subtypes as described in detail herein. Various subtypes of influenza virus exist and continue to be identified as new subtypes emerge. It will be understood by one skilled in the art that the methods and compositions provided herein may be adapted to utilize sequences of additional subtypes. Such variation is contemplated and encompassed within the methods and compositions provided herein.

Hemagglutinin Polypeptide Fusions with Thermostable Proteins

[050] In certain aspects, provided are HA polypeptide(s) comprising fusion polypeptides which comprise a HA polypeptide (or a portion or variant thereof) operably linked to a thermostable protein. Fusion polypeptides can be produced in any available expression system known in the art. In certain embodiments, fusion proteins are produced in a plant or portion thereof (*e.g.*, plant, plant cell, root, or sprout).

[051] Enzymes or other proteins that are not found naturally in human or animal cells can be particularly useful in fusion polypeptides as provided herein. For example, thermostable proteins that confer thermostability to a fusion product can be useful. Thermostability can allow a produced protein to maintain its conformation, and maintain produced protein at room temperature. This feature can facilitate easy, time efficient and cost effective recovery of a fusion polypeptide. A representative family of thermostable enzymes that can be used as described herein is the glucanohydrolase family. These enzymes specifically cleave 1,4-β glucosidic bonds that are adjacent to 1,3-β linkages in mixed linked polysaccharides (Hahn *et al.*, 1994 *Proc. Natl. Acad. Sci., USA*, 91:10417). Such enzymes are found in cereals, such as oat and barley, and are also found in a number of fungal and bacterial species, including *C. thermocellum* (Goldenkova *et al.*, 2002, *Mol. Biol.* 36:698). Thus, suitable thermostable proteins for use in fusion polypeptides as provided herein include glycosidase enzymes. Exemplary thermostable glycosidase proteins include those represented by GenBank accession numbers selected from those set forth in Table 2.

Exemplary thermostable enzymes for use in fusion proteins include beta-glucanase enzymes from *Clostridium thermocellum*, *Brevibacillus brevis*, and *Rhodthermus*

marinus. Representative fusion proteins can utilize modified thermostable enzymes isolated from *Clostridium thermocellum*, although any thermostable protein may be similarly utilized. Exemplary thermostable glycosidase proteins are listed in Table 2:

Table 2: Thermostable Glycosidase Proteins

Accession	Strain	Thermostable Protein Sequence
X63355	Beta-glucanase *Clostridium thermocellum*	5'MKNRVISLLMASLLLVLSVIVAPFYKAEAATVVNTPFVAV FSNFDSSQWEKADWANGSVFNCVWKPSQVTFSNGKMILTL DREYGGSYPYKSGEYRTKSFFGYGYYEVRMKAAKNVGIVS SFFTYTGPSDNNPWDEIDIEFLGKDTTKVQFNWYKNGVGGN EYLHNLGFDASQDFHTYGFEWRPDYIDFYVDGKKVYRGTR NIPVTPGKIMMNLWPGIGVDEWLGRYDGRTPLQAEYEYVK YYPNGVPQDNPTPTPTIAPSTPTNPNLPLKGDVNGDGHVNSS DYSLFKRYLLRVIDRFPVGDQSVADVNRDGRIDSTDLTMLK RYLIRAIPSL 3' (SEQ ID NO:36)
P37073	Beta-glucanase *Brevibacillus brevis*	5'MVKSKYLVFISVFSLLFGVFVVGFSHQGVKAEEERPMGTA FYESFDAFDDERWSKAGVWTNGQMFNATWYPEQVTADGL MRLTIAKKTTSARNYKAGELRTNDFYHYGLFEVSMKPAKV EGTVSSFFTYTGEWDWDGDPWDEIDIEFLGKDTTRIQFNYFT NGVGGNEFYYDLGFDASESFNTYAFEWREDSITWYVNGEA VHTATENIPQTPQKIMMNLWPGVGVDGWTGVFDGDNTPVY SYYDWVRYTPLQNYQIHQ 3' (SEQ ID NO:37)
P17989	Beta-glucanase *Fibrobacter succinogenes*	5'MNIKKTAVKSALAVAAAAAALTTNVSAKDFSGAELYTLE EVQYGKFEARMKMAAASGTVSSMFLYQNGSEIADGRPWVE VDIEVLGKNPGSFQSNIITGKAGAQKTSEKHHAVSPAADQAF HTYGLEWTPNYVRWTVDGQEVRKTEGGQVSNLTGTQGLR FNLWSSESAAWVGQFDESKLPLFQFINWVKVYKYTPGQGE GGSDFTLDWTDNFDTFDGSRWGKGDWTFDGNRVDLTDKNI YSRDGMLILALTRKGQESFNGQVPRDDEPAPQSSSSAPASSS SVPASSSSVPASSSSAFVPPSSSSATNAIHGMRTTPAVAKEHR NLVNAKGAKVNPNGHKRYRVNFEH 3' (SEQ ID NO:38)
P07883	Extracellular agarase *Streptomyces coelicolor*	5'MVNRRDLIKWSAVALGAGAGLAGPAPAAHAADLEWEQY PVPAAPGGNRSWQLLPSHSDDFNYTGKPQTFRGRWLDQHK DGWSGPANSLYSARHSWVADGNLIVEGRRAPDGRVYCGY VTSRTPVEYPLYTEVLMRVSGLKLSSNFWLLSRDDVNEIDVI ECYGNESLHGKHMNTAYHIFQRNPFTELARSQKGYFADGSY GYNGETGQVFGDGAGQPLLRNGFHRYGVHWISATEFDFYF NGRLVRRLNRSNDLRDPRSRFFDQPMHLILNTESHQWRVDR GIEPTDAELADPSINNIYYRWVRTYQAV 3' (SEQ ID NO:39)

P23903	Glucan endo-13-beta-glucosidase A1 *Bacillus circulans*	5'MKPSHFTEKRFMKKVLGLFLVVVMLASVGVLPTSKVQAA GTTVTSMEYFSPADGPVISKSGVGKASYGFVMPKFNGGSAT WNDVYSDVGVNVKVGNNWVDIDQAGGYIYNQNWGHWSD GGFNGYWFTLSATTEIQLYSKANGVKLEYQLVFQNINKTTIT AMNPTQGPQITASFTGGAGFTYPTFNNDSAVTYEAVADDLK VYVKPVNSSSWIDIDNNAASGWIYDHNFGQFTDGGGGYWF NVTESINVKLESKTSSANLVYTITFNEPTRNSYVITPYEGTTF TADANGSIGIPLPKIDGGAPIAKELGNFVYQININGQWVDLS NSSQSKFAYSANGYNNMSDANQWGYWADYIYGLWFQPIQ ENMQIRIGYPLNGQAGGNIGNNFVNYTFIGNPNAPRPDVSD QEDISIGTPTDPAIAGMNLIWQDEFNGTTLDTSKWNYETGY YLNNDPATWGWGNAELQHYTNSTQNVYVQDGKLNIKAMN DSKSFPQDPNRYAQYSSGKINTKDKLSLKYGRVDFRAKLPT GDGVWPALWMLPKDSVYGTWAASGEIDVMEARGRLPGSV SGTIHFGGQWPVNQSSGGDYHFPEGQTFANDYHVYSVVWE EDNIKWYVDGKFFYKVTNQQWYSTAAPNNPNAPFDEPFYLI MNLAVGGNFDGGRTPNASDIPATMQVDYVRVYKEQ 3' (SEQ ID NO:40)
P27051	Beta-glucanase *Bacillus licheniformis*	5'MSYRVKRMLMLLVTGLFLSLSTFAASASAQTGGSFYEPFN NYNTGLWQKADGYSNGNMFNCTWRANNVSMTSLGEMRL SLTSPSYNKFDCGENRSVQTYGYGLYEVNMKPAKNVGIVSS FFTYTGPTDGTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHE KIVNLGFDAANSYHTYAFDWQPNSIKWYVDGQLKHTATTQ IPQTPGKIMMNLWNGAGVDEWLGSYNGVTPLSRSLHWVRY TKR 3' (SEQ ID NO:41)
P45797	Beta-glucanase *Paenibacillus polymyxa Bacillus polymyxa*	5'MMKKKSWFTLMITGVISLFFSVSAFAGNVFWEPLSYFNSS TWQKADGYSNGQMFNCTWRANNVNFTNDGKLKLSLTSPA NNKFDCGEYRSTNNYGYGLYEVSMKPAKNTGIVSSFFTYTG PSHGTQWDEIDIEFLGKDTTKVQFNYYTNGVGGHEKIINLGF DASTSFHTYAFDWQPGYIKWYVDGVLKHTATTNIPSTPGKI MMNLWNGTGVDSWLGSYNGANPLYAEYDWVKYTSN 3' (SEQ ID NO:42)
P45798	Beta-glucanase *Rhodothermus marinus*	5'MCTMPLMKLKKMMRRTAFLLSVLIGCSMLGSDRSDKAPH WELVWSDEFDYSGLPDPEKWDYDVGGHGWGNQELQYYTR ARIENARVGGGVLIIEARHEPYEGREYTSARLVTRGKASWT YGRFEIRARLPSGRGTWPAIWMLPDRQTYGSAYWPDNGEID IMEHVGFNPDVVHGTVHTKAYNHLLGTQRGGSIRVPTARTD FHVYAIEWTPEEIRWFVDDSLYYRFPNERLTDPEADWRHWP FDQPFHLIMNIAVGGAWGGQQGVDPEAFPAQLVVDYVRVY RWVE 3' (SEQ ID NO:43)

P38645	Beta-glucosidase *Thermobis-pora bispora*	5'MTESAMTSRAGRGRGADLVAAVVQGHAAASDAAGDLSF PDGFIWGAATAAYQIEGAWREDGRGLWDVFSHTPGKVASG HTGDIACDHYHRYADDVRLMAGLGDRVYRFSVAWPRIVPD GSGPVNPAGLDFYDRLVDELLGHGITPYPTLYHWDLPQTLE DRGGWAARDTAYRFAEYALAVHRRLGDRVRCWITLNEPW VAAFLATHRGAPGAADVPRFRAVHHLLLGHGLGLRLRSAG AGQLGLTLSLSPVIEARPGVRGGGRRVDALANRQFLDPALR GRYPEEVLKIMAGHARLGHPGRDLETIHQPVDLLGVNYYSH VRLAAEGEPANRLPGSEGIRFERPTAVTAWPGDRPDGLRTL LLRLSRDYPGVGLIITENGAAFDDRADGDRVHDPERIRYLTA TLRAVHDAIMAGADLRGYFVWSVLDNFEWAYGYHKRGIV YVDYTTMRRIPRESALWYRDVVRRNGLRNGE 3' (SEQ ID NO:44)
P40942	Celloxy-lanase *Clostridium stercorar-ium*	5'MNKFLNKKWSLILTMGGIFLMATLSLIFATGKKAFNDQTS AEDIPSLAEAFRDYFPIGAAIEPGYTTGQIAELYKKHVNMLV AENAMKPASLQPTEGNFQWADADRIVQFAKENGMELRFHT LVWHNQTPTGFSLDKEGKPMVEETDPQKREENRKLLLQRL ENYIRAVVLRYKDDIKSWDVVNEVIEPNDPGGMRNSPWYQI TGTEYIEVAFRATREAGGSDIKLYINDYNTDDPVKRDILYEL VKNLLEKGVPIDGVGHQTHIDIYNPPVERIIESIKKFAGLGLD NIITELDMSIYSWNDRSDYGDSIPDYILTLQAKRYQELFDAL KENKDIVSAVVFWGISDKYSWLNGFPVKRTNAPLLFDRNFM PKPAFWAIVDPSRLRE 3' (SEQ ID NO:45)
P14002	Beta-glucosidase *Clostridium thermocell-um*	5'MAVDIKKIIKQMTLEEKAGLCSGLDFWHTKPVERLGIPSIM MTDGPHGLRKQREDAEIADINNSVPATCFPSAAGLACSWDR ELVERVGAALGEECQAENVSILLGPGANIKRSPLCGRNFEYF SEDPYLSSELAASHIKGVQSQGVGACLKHFAANNQEHRRMT VDTIVDERTLREIYFASFENAVKKARPWVVMCAYNKLNGE YCSENRYLLTEVLKNEWMHDGFVVSDWGAVNDRVSGLDA GLDLEMPTSHGITDKKIVEAVKSGKLSENILNRAVERILKVIF MALENKKENAQYDKDAHHRLARQAAAESMVLLKNEDDVL PLKKSGTIALIGAFVKKPRYQGSGSSHITPTRLDDIYEEIKKA GGDKVNLVYSEGYRLENDGIDEELINEAKKAASSSDVAVVF AGLPDEYESEGFDRTHMSIPENQNRLIEAVAEVQSNIVVVLL NGSPVEMPWIDKVKSVLEAYLGGQALGGALADVLFGEVNP SGKLAETFPVKLSHNPSYLNFPGEDDRVEYKEGLFVGYRYY DTKGIEPLFPFGHGLSYTKFEYSDISVDKKDVSDNSIINVSVK VKNVGKMAGKEIVQLYVKDVKSSVRRPEKELKGFEKVFLN PGEEKTVTFTLDKRAFAYYNTQIKDWHVESGEFLILIGRSSR DIVLKESVRVNSTVKIRKRFTVNSAVEDVMSDSSAAAVLGP VLKEITDALQIDMDNAHDMMAANIKNMPLRSLVGYSQGRL SEEMLEELVDKINNVE 3' (SEQ ID NO:46)

O33830	Alpha-glucosidase *Thermotoga maritima*	5'MPSVKIGIIGAGSAVFSLRLVSDLCKTPGLSGSTVTLMDID EERLDAILTIAKKYVEEVGADLKFEKTMNLDDVIIDADFVIN TAMVGGHTYLEKVRQIGEKYGYYRGIDAQEFNMVSDYYTF SNYNQLKYFVDIARKIEKLSPKAWYLQAANPIFEGTTLVTRT VPIKAVGFCHGHYGVMEIVEKLGLEEEKVDWQVAGVNHGI WLNRFRYNGGNAYPLLDKWIEEKSKDWKPENPFNDQLSPA AIDMYRFYGVMPIGDTVRNSSWRYHRDLETKKKWYGEPW GGADSEIGWKWYQDTLGKVTEITKKVAKFIKENPSVRLSDL GSVLGKDLSEKQFVLEVEKILDPERKSGEQHIPFIDALLNDN KARFVVNIPNKGIIHGIDDDVVVEVPALVDKNGIHPEKIEPPL PDRVVKYYLRPRIMRMEMALEAFLTGDIRIIKELLYRDPRTK SDEQVEKVIEEILALPENEEMRKHYLKR 3' (SEQ ID NO:47)
O43097	Xylanase *Thermomyces lanuginosus*	5'MVGFTPVALAALAATGALAFPAGNATELEKRQTTPNSEG WHDGYYYSWWSDGGAQATYTNLEGGTYEISWGDGGNLV GGKGWNPGLNARAIHFEGVYQPNGNSYLAVYGWTRNPLV EYYIVENFGTYDPSSGATDLGTVECDGSIYRLGKTTRVNAPS IDGTQTFDQYWSVRQDKRTSGTVQTGCHFDAWARAGLNV NGDHYYQIVATEGYFSSGYARITVADVG 3' (SEQ ID NO:48)
P54583	Endo-glucanase E1 *Acidothermus cellulolyticus*	5'MPRALRRVPGSRVMLRVGVVVAVLALVAALANLAVPRP ARAAGGGYWHTSGREILDANNVPVRIAGINWFGF*ETC*NYV VHGLWSRDYRSMLDQIKSLGYNTIRLPYSDDILKPGTMPNSI NFYQMNQDLQGLTSLQVMDKIVAYAGQIGLRIILDRHRPDC SGQSALWYTSSVSEATWISDLQALAQRYKGNPTVVGFDLH NEPHDPACWGCGDPSIDWRLAAERAGNAVLSVNPNLLIFVE GVQSYNGDSYWWGGNLQGAGQYPVVLNVPNRLVYSAHD YATSVYPQTWFSDPTFPNNMPGIWNKNWGYLFNQNIAPVW LGEFGTTLQSTTDQTWLKTLVQYLRPTAQYGADSFQWTFW SWNPDSGDTGGILKDDWQTVDTVKDGYLAPIKSSIFDPVGA SASPSSQPSPSVSPSPSPSPSASRTPTPTPTPTASPTPTLTPTATP TPTASPTPSPTAASGARCTASYQVNSDWGNGFTVTVAVTNS GSVATKTWTVSWTFGGNQTITNSWNAAVTQNGQSVTARN MSYNNVIQPGQNTTFGFQASYTGSNAAPTVACAAS 3' (SEQ ID NO:49)
P14288	β-galactosidase *Sulfolobus acidocaldarius*	5'MLSFPKGFKFGWSQSGFQSEMGTPGSEDPNSDWHVWVH DRENIVSQVVSGDLPENGPGYWGNYKRFHDEAEKIGLNAV RINVEWSRIFPRPLPKPEMQTGTDKENSPVISVDLNESKLRE MDNYANHEALSHYRQILEDLRNRGFHIVLNMYHWTLPIWL HDPIRVRRGDFTGPTGWLNSRTVYEFARFSAYVAWKLDDL ASEYATMNEPNVVWGAGYAFPRAGFPPNYLSFRLSEIAKW NIIQAHARAYDAIKSVSKKSVGIIYANTSYYPLRPQDNEAVEI AERLNRWSFFDSIIKGEITSEGQNVREDLRNRLDWIGVNYYT RTVVTKAESGYLTLPGYGDRCERNSLSLANLPTSDFGWEFF PEGLYDVLLKYWNRYGLPLYVMENGIADDADYQRPYYLVS HIYQVHRALNEGVDVRGYLHWSLADNYEWSSGFSMRFGLL KVDYLTKRLYWRPSALVYREITRSNGIPEELEHLNRVPPIKP LRH 3' (SEQ ID NO:50)

O52629	β-galacto-sidase *Pyrococcus woesei*	5'MFPEKFLWGVAQSGFQFEMGDKLRRNIDTNTDWWHWVR DKTNIEKGLVSGDLPEEGINNYELYEKDHEIARKLGLNAYRI GIEWSRIFPWPTTFIDVDYSYNESYNLIEDVKITKDTLEELDEI ANKREVAYYRSVINSLRSKGFKVIVNLNHFTLPYWLHDPIEA RERALTNKRNGWVNPRTVIEFAKYAAYIAYKFGDIVDMWS TFNEPMVVVELGYLAPYSGFPPGVLNPEAAKLAILHMINAH ALAYRQIKKFDTEKADKDSKEPAEVGIIYNNIGVAYPKDPN DSKDVKAAENDNFFHSGLFFEAIHKGKLNIEFDGETFIDAPY LKGNDWIGVNYYTREVVTYQEPMFPSIPLITFKGVQGYGYA CRPGTLSKDDRPVSDIGWELYPEGMYDSIVEAHKYGVPVYV TENGIADSKDILRPYYIASHIKMTEKAFEDGYEVKGYFHWA LTDNFEWALGFRMRFGLYEVNLITKERIPREKSVSIFREIVAN NGVTKKIEEELLRG 3' (SEQ ID NO:51)
P29094	Oligo-16-glucosidase *Geobacillus thermoglucosidasius*	5'MERVWWKEAVVYQIYPRSFYDSNGDGIGDIRGIIAKLDYL KELGVDVVWLSPVYKSPNDDNGYDISDYRDIMDEFGTMAD WKTMLEEMHKRGIKLVMDLVVNHTSDEHPWFIESRKSKDN PYRDYYIWRPGKNGKEPNNWESVFSGSAWEYDEMTGEYYL HLFSKKQPDLNWENPKVRREVYEMMKFWLDKGVDGFRMD VINMISKVPELPDGEPQSGKKYASGSRYYMNGPRVHEFLQE MNREVLSKYDIMTVGETPGVTPKEGILYTDPSRRELNMVFQ FEHMDLDSGPGGKWDIRPWSLADLKKTMTKWQKELEGKG WNSLYLNNHDQPRAVSRFGDDGKYRVESAKMLATFLHMM QGTPYIYQGEEIGMTNVRFPSIEDYRDIETLNMYKERVEEYG EDPQEVMEKIYYKGRDNARTPMQWDDSENAGFTAGTPWIP VNPNYKEINVKAALEDPNSVFHYYKKLIQLRKQHDIIVYGT YDLILEDDPYIYRYTRTLGNEQLIVITNFSEKTPVFRLPDHIIY KTKELLISNYDVDEAEELKEIRLRPWEARVYKIRLP 3' (SEQ ID NO:52)
P49067	Alpha-amylase *Pyrococcus furiosus*	5'MGDKINFIFGIHNHQPLGNFGWVFEEAYEKCYWPFLETLE EYPNMKVAIHTSGPLIEWLQDNRPEYIDLLRSLVKRGQVEIV VAGFYEPVLASIPKEDRIEQIRLMKEWAKSIGFDARGVWLTE RVWQPELVKTLKESGIDYVIVDDYHFMSAGLSKEELYWPY YTEDGGEVIAVFPIDEKLRYLIPFRPVDKVLEYLHSLIDGDES KVAVFHDDGEKFGIWPGTYEWVYEKGWLREFFDRISSDEKI NLMLYTEYLEKYKPRGLVYLPIASYFEMSEWSLPAKQARLF VEFVNELKVKGIFEKYRVFVRGGIWKNFFYKYPESNYMHK RMLMVSKLVRNNPEARKYLLRAQCNDAYWHGLFGGVYLP HLRRAIWNNLIKANSYVSLGKVIRDIDYDGFEEVLIENDNFY AVFKPSYGGSLVEFSSKNRLVNYVDVLARRWEHYHGYVES QFDGVASIHELEKKIPDEIRKEVAYDKYRRFMLQDHVVPLG TTLEDFMFSRQQEIGEFPRVPYSYELLDGGIRLKREHLGIEVE KTVKLVNDGFEVEYIVNNKTGNPVLFAVELNVAVQSIMESP GVLRGKEIVVDDKYAVGKFALKFEDEMEVWKYPVKTLSQS ESGWDLIQQGVSYIVPIRLEDKIRFKLKFEEASG 3' (SEQ ID NO:53)

JC7532	Cellulase *Bacillus* species	5'MMLRKKTKQLISSILILVLLLSLFPAALAAEGNTREDNFKH LLGNDNVKRPSEAGALQLQEVDGQMTLVDQHGEKIQLRGM STHGLQWFPEILNDNAYKALSNDWDSNMIRLAMYVGENGY ATNPELIKQRVIDGIELAIENDMYVIVDWHVHAPGDPRDPV YAGAKDFFREIAALYPNNPHIIYELANEPSSNNNGGAGIPNN EEGWKAVKEYADPIVEMLRKSGNADDNIIIVGSPNWSQRPD LAADNPIDDHHTMYTVHFYTGSHAASTESYPSETPNSERGN VMSNTRYALENGVAVFATEWGTSQASGDGGPYFDEADVWI EFLNENNISWANWSLTNKNEVSGAFTPFELGKSNATNLDPG PDHVWAPEELSLSGEYVRARIKGVNYEPIDRTKYTKVLWDF NDGTKQGFGVNSDSPNKELIAVDNENNTLKVSGLDVSNDVS DGNFWANARLSANGWGKSVDILGAEKLTMDVIVDEPTTVA IAAIPQSSKSGWANPERAVRVNAEDFVQQTDGKYKAGLTIT GEDAPNLKNIAFHEEDNNMNNIILFVGTDAADVIYLDNIKVI GTEVEIPVVHDPKGEAVLPSVFEDGTRQGWDWAGESGVKT ALTIEEANGSNALSWEFGYPEVKPSDNWATAPRLDFWKSDL VRGENDYVAFDFYLDPVRATEGAMNINLVFQPPTNGYWVQ APKTYTINFDELEEANQVNGLYHYEVKINVRDITNIQDDTLL RNMMIIFADVESDFAGRVFVDNVRFEGAATTEPVEPEPVDP GEETPPVDEKEAKKEQKEAEKEEKEAVKEEKKEAKEEKKA VKNEAKKK 3' (SEQ ID NO:54)
Q60037	Xylanase A *Thermotoga maritima*	5'MQVRKRRGLLDVSTAVLVGILAGFLGVVLAASGVLSFGK EASSKGDSSLETVLALSFEGTTEGVVPFGKDVVLTASQDVA ADGEYSLKVENRTSPWDGVEIDLTGKVKSGADYLLSFQVY QSSDAPQLFNVVARTEDEKGERYDVILDKVVVSDHWKEILV PFSPTFEGTPAKYSLIIVASKNTNFNFYLDKVQVLAPKESGPK VIYETSFENGVGDWQPRGDVNIEASSEVAHSGKSSLFISNRQ KGWQGAQINLKGILKTGKTYAFEAWVYQNSGQDQTIIMTM QRKYSSDASTQYEWIKSATVPSGQWVQLSGTYTIPAGVTVE DLTLYFESQNPTLEFYVDDVKIVDTTSAEIKIEMEPEKEIPAL KEVLKDYFKVGVALPSKVFLNPKDIELITKHFNSITAENEMK PESLLAGIENGKLKFRFETADKYIQFVEENGMVIRGHTLVW HNQTPDWFFKDENGNLLSKEAMTERLKEYIHTVVGHFKGK VYAWDVVNEAVDPNQPDGLRRSTWYQIMGPDYIELAFKFA READPDAKLFYNDYNTFEPRKRDIIYNLVKDLKEKGLIDGIG MQCHISLATDIKQIEEAIKKFSTIPGIEIHITELDMSVYRDSSSN YPEAPRTALIEQAHKMMQLFEIFKKYSNVITNVTFWGLKDD YSWRATRRNDWPLIFDKDHQAKLAYWAIVAPEVLPPLPKES RISEGEAVVVGMMDDSYLMSKPIEILDEEGNVKATIRAVWK DSTIYIYGEVQDKTKKPAEDGVAIFINPNNERTPYLQPDDTY AVLWTNWKTEVNREDVQVKKFVGPGFRRYSFEMSITIPGVE FKKDSYIGFDAAVIDDGKWYSWSDTTNSQKTNTMNYGTLK LEGIMVATAKYGTPVIDGEIDEIWNTTEEIETKAVAMGSLDK NATAKVRVLWDENYLYVLAIVKDPVLNKDNSNPWEQDSV EIFIDENNHKTGYYEDDDAQFRVNYMNEQTFGTGGSPARFK TAVKLIEGGYIVEAAIKWKTIKPTPNTVIGFNIQVNDANEKG QRVGIISWSDPTNNSWRDPSKFGNLRLIK 3' (SEQ ID NO:55)

P33558	Xylanase A *Clostridium stercorarium*	5'MKRKVKKMAAMATSIIMAIMIILHSIPVLAGRIIYDNETGT HGGYDYELWKDYGNTIMELNDGGTFSCQWSNIGNALFRKG RKFNSDKTYQELGDIVVEYGCDYNPNGNSYLCVYGWTRNP LVEYYIVESWGSWRPPGATPKGTITQWMAGTYEIYETTRVN QPSIDGTATFQQYWSVRTSKRTSGTISVTEHFKQWERMGMR MGKMYEVALTVEGYQSSGYANVYKNEIRIGANPTPAPSQSP IRRDAFSIIEAEEYNSTNSSTLQVIGTPNNGRGIGYIENGNTVT YSNIDFGSGATGFSATVATEVNTSIQIRSDSPTGTLLGTLYVS STGSWNTYQTVSTNISKITGVHDIVLVFSGPVNVDNFIFSRSS PVPAPGDNTRDAYSIIQAEDYDSSYGPNLQIFSLPGGGSAIGY IENGYSTTYKNIDFGDGATSVTARVATQNATTIQVRLGSPSG TLLGTIYVGSTGSFDTYRDVSATISNTAGVKDIVLVFSGPVN VDWFVFSKSGT 3' (SEQ ID NO:56)
P05117	Polygalacturonase-2 precursor *Solanum lycopersicum*	5'MVIQRNSILLLIIIFASSISTCRSNVIDDNLFKQVYDNILEQEF AHDFQAYLSYLSKNIESNNNIDKVDKNGIKVINVLSFGAKG DGKTYDNIAFEQAWNEACSSRTPVQFVVPKNKNYLLKQITF SGPCRSSISVKIFGSLEASSKISDYKDRRLWIAFDSVQNLVVG GGGTINGNGQVWWPSSCKINKSLPCRDAPTALTFWNCKNL KVNNLKSKNAQQIHIKFESCTNVVASNLMINASAKSPNTDG VHVSNTQYIQISDTIIGTGDDCISIVSGSQNVQATNITCGPGH GISIGSLGSGNSEAYVSNVTVNEAKIIGAENGVRIKTWQGGS GQASNIKFLNVEMQDVKYPIIIDQNYCDRVEPCIQQFSAVQV KNVVYENIKGTSATKVAIKFDCSTNFPCEGIIMENINLVGESG KPSEATCKNVHFNNAEHVTPHCTSLEISEDEALLYNY 3' (SEQ ID NO:57)
P04954	Cellulase D *Clostridium thermocellum*	5'MSRMTLKSSMKKRVLSLLIAVVFLSLTGVFPSGLIETKVSA AKITENYQFDSRIRLNSIGFIPNHSKKATIAANCSTFYVVKED GTIVYTGTATSMFDNDTKETVYIADFSSVNEEGTYYLAVPG VGKSVNFKIAMNVYEDAFKTAMLGMYLLRCGTSVSATYNG IHYSHGPCHTNDAYLDYINGQHTKKDSTKGWHDAGDYNK YVVNAGITVGSMFLAWEHFKDQLEPVALEIPEKNNSIPDFLD ELKYEIDWILTMQYPDGSGRVAHKVSTRNFGGFIMPENEHD ERFFVPWSSAATADFVAMTAMAARIFRPYDPQYAEKCINAA KVSYEFLKNNPANVFANQSGFSTGEYATVSDADDRLWAAA EMWETLGDEEYLRDFENRAAQFSKKIEADFDWDNVANLG MFTYLLSERPGKNPALVQSIKDSLLSTADSIVRTSQNHGYGR TLGTTYYWGCNGTVVRQTMILQVANKISPNNDYVNAALDA ISHVFGRNYYNRSYVTGLGINPPMNPHDRRSGADGIWEPWP GYLVGGGWPGPKDWVDIQDSYQTNEIAINWNAALIYALAG FVNYNSPQNEVLYGDVNDDGKVNSTDLTLLKRYVLKAVST LPSSKAEKNADVNRDGRVNSSDVTILSRYLIRVIEKLPI 3' (SEQ ID NO:58)

Q4J929	N-glycosylase *Sulfolobus acidocaldarius*	5'MLRSLVLNEKLRARVLERAEEFLLNNKADEEVWFRELVL CILTSNSSFISAYKSMNYILDKILYMDEKEISILLQESGYRFYN LKAKYLYRAKNLYGKVKKTIKEIADKDQMQAREFIATHIYG IGYKEASHFLRNVGYLDLAIIDRHILRFINNLGIPIKLKSKREY LLAESLLRSIANNLNVQVGLLDLFIFFKQTNTIVK 3' (SEQ ID NO:59)
O33833	Beta-fructosidase *Thermotoga maritima*	5'MFKPNYHFFPITGWMNDPNGLIFWKGKYHMFYQYNPRKP EWGNICWGHAVSDDLVHWRHLPVALYPDDETHGVFSGSA VEKDGKMFLVYTYYRDPTHNKGEKETQCVAMSENGLDFV KYDGNPVISKPPEEGTHAFRDPKVNRSNGEWRMVLGSGKD EKIGRVLLYTSDDLFHWKYEGVIFEDETTKEIECPDLVRIGE KDILIYSITSTNSVLFSMGELKEGKLNVEKRGLLDHGTDFYA AQTFFGTDRVVVIGWLQSWLRTGLYPTKREGWNGVMSLPR ELYVENNELKVKPVDELLALRKRKVFETAKSGTFLLDVKEN SYEIVCEFSGEIELRMGNESEEVVITKSRDELIVDTTRSGVSG GEVRKSTVEDEATNRIRAFLDSCSVEFFFNDSIAFSFRIHPEN VYNILSVKSNQVKLEVFELENIWL 3' (SEQ ID NO:60)
P49425	Endo-1,4-beta-mannosidase *Rhodothermus marinus*	5'MAGPHRSRAAGPPPFAVDEHVALEMVAFRGEVFAGHGLL ADQRLIAHTGRPALNAQRITQQKQRDQCRGQRHRHHQGGR NLRKAHRTFHEHQSTQDQAHDAPHGQQAKTGHEGLGHEH AQAQHQQGQSNVVDRQDGEPVEAQHQKDGAQRAGNAPA GRVELEQQPVEAQHQQQEGDVRIGKRRQNAFAPPALDHVH GGPGRLQRHGLAVERHVPAVQQHQQRVQRGRQQIDHVLG HGLPGRQRLAFRDGPRRPVGVASPVLGQRPCPGHRIVQNLF RHGIDPCRVGRCRRSPSELHGMGCADVRARGHGRHMRGQR DEHPGRGRPCARRRHVDDDRDRTPQEKLYDVARGLDEPAR RVHFDDEADRSVFRGLAQPAPDEPEGRRRDRLVLQRQSVN HRRGRLSRHRQQHQPQQQRPHGNQAFLGKYEKRRRKPTAC LKSLRRFPDKDAPVLYFVNQLEKTKRRMTLLLVWLIFTGVA GEIRLEAEDGELLGVAVDSTLTGYSGRGYVTGFDAPEDSVR FSFEAPRGVYRVVFGVSFSSRFASYALRVDDWHQTGSLIKR GGGFFEASIGEIWLDEGAHTMAFQLMNGALDYVRLEPVSY GPPARPPAQLSDSQATASAQALFAFLLSEYGRHILAGQQQNP YRRDFDAINYVRNVTGKEPALVSFDLIDYSPTREAHGVVHY QTPEDWIAWAGRDGIVSLMWHWNAPTDLIEDPSQDCYWW YGFYTRCTTFDVAAALADTSSERYRLLLRDIDVIAAQLQKF QQADIPVLWRPLHEAAGGWFWWGAKGPEPFKQLWRLLYE RLVHHHGLHNLIWVYTHEPGAAEWYPGDAYVDIVGRDVY ADDPDALMRSDWNELQTLFGGRKLVALTETGTLPDVEVITD YGIWWSWFSIWTDPFLRDVDPDRLTRVYHSERVLTRDELPD WRSYVLHATTVQPAGDLALAVYPNPGAGRLHVEVGLPVAA PVVVEVFNLLGQRVFQYQAGMQPAGLWRRAFELALAPGV YLVQVRAGNLVARRRWVSVR 3' (SEQ ID NO:61)

P06279	Alpha-amylase *Geobacillus stearotherm-ophilus*	5'MLTFHRIIRKGWMFLLAFLLTALLFCPTGQPAKAAAPFNG TMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPA YKGTSRSDVGYGVYDLYDLGEFNQKGAVRTKYGTKAQYL QAIQAAHAAGMQVYADVVFDHKGGADGTEWVDAVEVNP SDRNQEISGTYQIQAWTKFDFPGRGNTYSSFKWRWYHFDG VDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLMYA DLDMDHPEVVTELKSWGKWYVNTTNIDGFRLDAVKHIKFS FFPDWLSDVRSQTGKPLFTVGEYWSYDINKLHNYIMKTNGT MSLFDAPLHNKFYTASKSGGTFDMRTLMTNTLMKDQPTLA VTFVDNHDTEPGQALQSWVDPWFKPLAYAFILTRQEGYPC VFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQHDYLDH SDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHA GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKT TVSTIAWSITTRPWTDEFVRWTEPRLVAWP 3' (SEQ ID NO:62)

P45702 P45703 P40943	Xylanase *Geobacillus stearotherm-ophilus*	5'MPTNLFFNAHHSPVGAFASFTLGFPGKSGGLDLELARPPR QNVLIGVESLHESGLYHVLPFLETAEEDESKRYDIENPDPNP QKPNILIPFAKEEIQREFHVATDTWKAGDLTFTIYSPVKAVP NPETADEEELKLALVPAVIVEMTIDNTNGTRARRAFFGFEGT DPYTSMRRIDDTCPQLRGVGQGRILSIVSKDEGVRSALHFSM EDILTAQLEENWTFGLGKVGALIVDVPAGEKKTYQFAVCFY RGGYVTAGMDASYFYTRFFQNIEEVGLYALEQAEVLKEQSF RSNKLIEKEWLSDDQTFMMAHAIRSYYGNTQLLEHEGKPIW VVNEGEYRMMNTFDLTVDQLFFELKLNPWTVKNVLDLYVE RYSYEDRVRFPGEETEYPSGISFTHDMGVANTFSRPHYSSYE LYGISGCFSHMTHEQLVNWVLCAAVYIEQTKDWAWRDKR LAILEQCLESMVRRDHPDPEQRNGVMGLDSTRTMGGAEITT YDSLDVSLGQARNNLYLAGKCWAAYVALEKLFRDVGKEE LAALAGEQAEKCAATIVSHVTDDGYIPAIMGEGNDSKIIPAIE GLVFPYFTNCHEALDENGRFGAYIQALRNHLQYVLREGICL FPDGGWKISSTSNNSWLSKIYLCQFIARHILGWEWDEQGKR ADAAHVAWLTHPTLSIWSWSDQIIAGEITGSKYYPRGVTSIL WLEEGE 3' (SEQ ID NO:63) 5'MCSSIPSLREVFANDFRIGAAVNPVTLEAQQSLLIRHVNSL TAENHMKFEHLQPEEGRFTFDIAIKSSTSPFSSHGVRGHTLV WHNQTPSWVFQDSQGHFVGRDVLLERMKSHISTVVQRYKG KVYCWDVINEAVADEGSEWLRSSTWRQIIGDDFIQQAFLYA HEADPEALLFYNDYNECFPEKREKIYTLVKSLRDKGIPIHGIG MQAHWSLNRPTLDEIRAAIERYASLGVILHITELDISMFEFDD HRKDLAAPTNEMVERQAERYEQIFSLFKEYRDVIQNVTFWG IADDHTWLDHFPVQGRKNWPLLFDEQHNPKPAFWRVVNI 3' (SEQ ID NO:64) 5'MRNVVRKPLTIGLALTLLLPMGMTATSAKNADSYAKKPH ISALNAPQLDQRYKNEFTIGAAVEPYQLQNEKDVQMLKRHF NSIVAENVMKPISIQPEEGKFNFEQADRIVKFAKANGMDIRF HTLVWHSQVPQWFFLDKEGKPMVNETDPVKREQNKQLLL KRLETHIKTIVERYKDDIKYWDVVNEVVGDDGKLRNSPWY QIAGIDYIKVAFQAARKYGGDNIKLYMNDYNTEVEPKRTAL YNLVKQLKEEGVPIDGIGHQSHIQIGWPSEAEIEKTINMFAAL GLDNQITELDVSMYGWPPRAYPTYDAIPKQKFLDQAARYD RLFKLYEKLSDKISNVTFWGIADNHTWLDSRADVYYDANG NVVVDPNAPYAKVEKGKGKDAPFVFGPDYKVKPAYWAIID HK 3' (SEQ ID NO:65)

P09961	Alpha-amylase 1 *Dictyoglomus thermophilum*	5'MTKSIYFSLGIHNHQPVGNFDFVIERAYEMSYKPLINFFFK HPDFPINVHFSGFLLLWLEKNHPEYFEKLKIMAERGQIEFVS GGFYEPILPIIPDKDKVQQIKKLNKYIYDKFGQTPKGMWLAE RVWEPHLVKYIAEAGIEYVVVDDAHFFSVGLKEEDLFGYYL MEEQGYKLAVFPISMKLRYLIPFADPEETITYLDKFASEDKS KIALLFDDGEKFGLWPDTYRTVYEEGWLETFVSKIKENFLL VTPVNLYTYMQRVKPKGRIYLPTASYREMMEWVLFPEAQK ELEELVEKLKTENLWDKFSPYVKGGFWRNFLAKYDESNHM QKKMLYVWKKVQDSPNEEVKEKAMEEVFQGQANDAYWH GIFGGLYLPHLRTAIYEHLIKAENYLENSEIRFNIFDFDCDGN DEIIVESPFFNLYLSPNHGGSVLEWDFKTKAFNLTNVLTRRK EAYHSKLSYVTSEAQGKSIHERWTAKEEGLENILFYDNHRR VSFTEKIFESEPVLEDLWKDSSRLEVDSFYENYDYEINKDEN KIRVLFSGVFRGFELCKSYILYKDKSFVDVVYEIKNVSETPIS LNFGWEINLNFLAPNHPDYYFLIGDQKYPLSSFGIEKVNNW KIFSGIGIELECVLDVEASLYRYPIETVSLSEEGFERVYQGSAL IHFYKVDLPVGSTWRTTIRFWVK 3' (SEQ ID NO:66)
Q60042	Xylanase A *Thermotoga neapolitana*	5'MRKKRRGFLNASTAVLVGILAGFLGVVLAATGALGFAVR ESLLLKQFLFLSFEGNTDGASPFGKDVVVTASQDVAADGEY SLKVENRTSVWDGVEIDLTGKVNTGTDYLLSFHVYQTSDSP QLFSVLARTEDEKGERYKILADKVVVPNYWKEILVPFSPTFE GTPAKFSLIITSPKKTDFVFYVDNVQVLTPKEAGPKVVYETS FEKGIGDWQPRGSDVKISISPKVAHSGKKSLFVSNRQKGWH GAQISLKGILKTGKTYAFEAWVYQESGQDQTIIMTMQRKYS SDSSTKYEWIKAATVPSGQWVQLSGTYTIPAGVTVEDLTLY FESQNPTLEFYVDDVKVVDTTSAEIKLEMNPEEEIPALKDVL KDYFRVGVALPSKVFINQKDIALISKHSNSSTAENEMKPDSL LAGIENGKLKFRFETADKYIEFAQQNGMVVRGHTLVWHNQ TPEWFFKDENGNLLSKEEMTERLREYIHTVVGHFKGKVYA WDVVNEAVDPNQPDGLRRSTWYQIMGPDYIELAFKFAREA DPNAKLFYNDYNTFEPKKRDIIYNLVKSLKEKGLIDGIGMQC HISLATDIRQIEEAIKKFSTIPGIEIHITELDISVYRDSTSNYSEA PRTALIEQAHKMAQLFKIFKKYSNVITNVTFWGLKDDYSWR ATRRNDWPLIFDKDYQAKLAYWAIVAPEVLPPLPKESKISEG EAVVVGMMDDSYMMSKPIEIYDEEGNVKATIRAIWKDSTIY VYGEVQDATKKPAEDGVAIFINPNNERTPYLQPDDTYVVLW TNWKSEVNREDVEVKKFVGPGFRRYSFEMSITIPGVEFKKD SYIGFDVAVIDDGKWYSWSDTTNSQKTNTMNYGTLKLEGV MVATAKYGTPVIDGEIDDIWNTTEEIETKSVAMGSLEKNAT AKVRVLWDEENLYVLAIVKDPVLNKDNSNPWEQDSVEIFID ENNHKTGYYEDDDAQFRVNYMNEQSFGTGASAARFKTAV KLIEGGYIVEAAIKWKTIKPSPNTVIGFNVQVNDANEKGQRV GIISWSDPTNNSWRDPSKFGNLRLIK 3' (SEQ ID NO:67)

AAN05438 AAN05439	Beta-glycosidase *Thermus thermo-philus*	5'MDDHAEKFLWGVATSAYQIEGATQEDGRGPSIWDAFARR PGAIRDGSTGEPACDHYRRYEEDIALMQSLGVRAYRFSVAW PRILPEGRGRINPKGLAFYDRLVDRLLASGITPFLTLYHWDLP LALEERGGWRSRETAFAFAEYAEAVARALADRVPFFATLNE PWCSAFLGHWTGEHAPGLRNLEAALRAAHHLLLGHGLAVE ALRAAGARRVGIVLNFAPAYGEDPEAVDVADRYHNRYFLD PILGKGYPESPFRDPPPVPILSRDLELVARPLDFLGVNYYAPV RVAPGTGTLPVRYLPPEGPATAMGWEVYPEGLHHLLKRLG REVPWPLYVTENGAAYPDLWTGEAVVEDPERVAYLEAHVE AALRAREEGVDLRGYFVWSLMDNFEWAFGYTRRFGLYYV DFPSQRRIPKRSALWYRERIARAQT 3' (SEQ ID NO:68) 5'MTENAEKFLWGVATSAYQIEGATQEDGRGPSIWDAFAQR PGAIRDGSTGEPACDHYRRYEEDIALMQSLGVRAYRFSVAW PRILPEGRGRINPKGLAFYDRLVDRLLASGITPFLTLYHWDLP LALEERGGWRSRETAFAFAEYAEAVARALADRVPFFATLNE PWCSAFLGHWTGEHAPGLRNLEAALRAAHHLLLGHGLAVE ALRAAGARRVGIVLNFAPAYGEDPEAVDVADRYHNRFFLD PILGKGYPESPFRDPPPVPILSRDLELVARPLDFLGVNYYAPV RVAPGTGTLPVRYLPPEGPATAMGWEVYPEGLYHLLKRLG REVPWPLYVTENGAAYPDLWTGEAVVEDPERVAYLEAHVE AALRAREEGVDLRGYFVWSLMDNFEWAFGYTRRFGLYYV DFPSQRRIPKRSALWYRERIARAQT 3' (SEQ ID NO:69)
AAN05437	Sugar permease *Thermus thermo-philus*	5'MAQVGRGASPLSRARVPPLPHPLDGEHLPHDPAGGGHGK ASSQDAPVGQLPGHLARPAFFHYLKNSFLVCSLTTVFALAV ATFAGYALARFRFPGAELFGGSVLVTQVIPGILFLIPIYIMYIY VQNWVRSALGLEVRLVGSYGGLVFTYTAFFVPLSIWILRGF FASIPKELEEAAMVDGATPFQAFHRVILPLALPGLAATAVYI FLTAWDELLFAQVLTTEATATVPVGIRNFVGNYQNRYDLV MAAATVATLPVLVLFFFVQRQLIQGLTAGAVKG 3' (SEQ ID NO:70)
AAN05440	Beta-glycosidase *Thermus filiformis*	5'MAENAEKFLWGVATSAYQIEGATQEDGRGPSIWDTFARR PGAIRDGSTGEPACDHYHRYEEDIALMQSLGVGVYRFSVA WPRILPEGRGRINPKGLAFYDRLVDRLLAAGITPFLTLYHWD LPQALEDRGGWRSRETAFAFAEYAEAVARALADRVPFFATL NEPWCSAFLGHWTGEHAPGLRNLEAALRAAHHLLLGHGLA VEALRAAGAKRVGIVLNFAPVYGEDPEAVDVADRYHNRYF LDPILGRGYPESPFQDPPPTPNLSRDLELVARPLDFLGVNYY APVRVAPGTGPLPVRYLPPEGPVTAMGWEVYPEGLYHLLK RLGREVPWPLYITENGAAYPDLWTGEAVVEDPERVAYLEA HVEAALRAREEGVDLRGYFVWSLMDNFEWAFGYTRRFGL YYVDFPSQRRIPKRSALWYRERIARAQL 3' (SEQ ID NO:71)

AAD43138	Beta-glycosidase *Thermo-sphaera aggregans*	5'MKFPKDFMIGYSSSPFQFEAGIPGSEDPNSDWWVWVHDPE NTAAGLVSGDFPENGPGYWNLNQNDHDLAEKLGVNTIRVG VEWSRIFPKPTFNVKVPVERDENGSIVHVDVDDKAVERLDE LANKEAVNHYVEMYKDWVERGRKLILNLYHWPLPLWLHN PIMVRRMGPDRAPSGWLNEESVVEFAKYAAYIAWKMGELP VMWSTMNEPNVVYEQGYMFVKGGFPPGYLSLEAADKARR NMIQAHARAYDNIKRFSKKPVGLIYAFQWFELLEGPAEVFD KFKSSKLYYFTDIVSKGSSIINVEYRRDLANRLDWLGVNYYS RLVYKIVDDKPIILHGYGFLCTPGGISPAENPCSDFGWEVYPE GLYLLLKELYNRYGVDLIVTENGVSDSRDALRPAYLVSHVY SVWKAANEGIPVKGYLHWSLTDNYEWAQGFRQKFGLVMV DFKTKKRYLRPSALVFREIATHNGIPDELQHLTLIQ 3' (SEQ ID NO:72)

[052] While sequences of exemplary thermostable polypeptides are provided herein, it will be appreciated that any sequence exhibiting thermostability may be employed. In some embodiments, a thermostable polypeptide can have an amino acid sequence with about 60%, about 70%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to an amino acid sequence selected from the group consisting of SEQ ID NOS:36-72. In some embodiments, such a thermostable polypeptide can retain thermostability.

[053] In some embodiments, a thermostable polypeptide can have an amino acid sequence that comprises about 100 contiguous amino acids of a sequence selected from the group consisting of SEQ ID NOS:36-72. In some embodiments, a thermostable polypeptide can have an amino acid sequence with about 60%, about 70%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to a contiguous stretch of about 100 amino acids from a sequence selected from the group consisting of SEQ ID NOS:36-72.

[054] In some embodiments, a thermostable polypeptide can have an amino acid sequence comprising about 150, about 200, about 250, about 300, about 350, about 400, about 450, about 500, about 550, about 600, about 650, about 700, or more than 700 contiguous amino acids of a sequence selected from the group consisting of SEQ ID NOS:36-72. In some embodiments, a thermostable polypeptide can have an amino acid sequence with about 60%, about 70%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or 100% sequence identity to a contiguous stretch of about 150, 200, 250, 300, 350, or more than 350 amino acids from a sequence selected from the group consisting of SEQ ID NO:36-72.

[055] When designing fusion proteins and polypeptides, it typically is desirable to preserve immunogenicity of the antigen. Still further, it is desirable in certain aspects to provide constructs which provide thermostability of a fusion protein. This feature facilitates easy, time efficient and cost effective recovery of a target antigen. In certain aspects, antigen fusion partners may be selected which provide additional advantages, including enhancement of immunogenicity, potential to incorporate multiple vaccine determinants, yet lack prior immunogenic exposure to vaccination subjects. Further beneficial qualities of fusion peptides of interest include proteins which provide ease of manipulation for incorporation of one or more antigens, as well as proteins which have potential to confer ease of production, purification, and/or formulation for vaccine preparations. One of ordinary skill in the art will appreciate that three dimensional presentation can affect each of these beneficial characteristics. Preservation of immunity or preferential qualities therefore may affect, for example, choice of fusion partner and/or choice of fusion location (*e.g.*, N-terminus, C-terminus, internal, combinations thereof). Alternatively or additionally, preferences may affect length of segment selected for fusion, whether it be length of antigen or length of fusion partner selected.

[056] As described herein, a variety of antigens can be fused with a thermostable protein. For example, the thermostable carrier molecule LicB, also referred to as lichenase, can be used for production of fusion proteins. LicB is 1,3-1,4-β glucanase (LicB) from *Clostridium thermocellum*, and has the following amino acid sequence (also set forth in EMBL accession: X63355 [gi:40697]):

MKNRVISLLMASLLLVLSVIVAPFYKAEAATVVNTPFVAVFSNFDSSQ
WEKADWANGSVFNCVWKPSQVTFSNGKMILTLDREYGGSYPYKSGE
YRTKSFFGYGYYEVRMKAAKNVGIVSSFFTYTGPSDNNPWDEIDIEFL
GKDTTKVQFNWYKNGVGGNEYLHNLGFDASQDFHTYGFEWRPDYID
FYVDGKKVYRGTRNIPVTPGKIMMNLWPGIGVDEWLGRYDGRTPLQ
AEYEYVKYYPNGVPQDNPTPTPTIAPSTPTNPNLPLKGDVNGDGHVNS
SDYSLFKRYLLRVIDRFPVGDQSVADVNRDGRIDSTDLTMLKRYLIRAI
PSL (SEQ ID NO:36).

[057] LicB belongs to a family of globular proteins. Based on the three dimensional structure of LicB, its N- and C-termini are situated close to each other on the surface, in close proximity to the active domain. LicB also has a loop structure exposed on the surface that is located far from the active domain. We have generated constructs such that the loop structure and N- and C-termini of protein can be used as insertion sites for HA polypeptides. HA

polypeptides can be expressed as N- or C-terminal fusions or as inserts into the surface loop. Importantly, LicB maintains its enzymatic activity at low pH and at high temperature (up to 75°C). Thus, use of LicB as a carrier molecule contributes advantages, including likely enhancement of target specific immunogenicity, potential to incorporate multiple vaccine determinants, and straightforward formulation of vaccines that may be delivered nasally, orally or parenterally. Furthermore, production of LicB fusions in plants should reduce the risk of contamination with animal or human pathogens. See examples provided herein.

[058] Fusion proteins comprising HA polypeptides can be produced in any of a variety of expression systems, including both *in vitro* and *in vivo* systems. One skilled in the art will readily appreciate that optimization of nucleic acid sequences for a particular expression system is often desirable. For example, an exemplary optimized sequence for expression of HA polypeptide-LicB fusions in plants is provided, and is shown in SEQ ID NO:73:

5'**MGFVLFSQLPSFLLVSTLLLFLVISHSCRAQN**GGSYPYKSGEYRTK
SFFGYGYYEVRMKAAKNVGIVSSFFTYTGPSDNNPWDEIDIEFLGKDT
TKVQFNWYKNGVGGNEYLHNLGFDASQDFHTYGFEWRPDYIDFYVD
GKKVYRGTRNIPVTPGKIMMNLWPGIGVDEWLGRYDGRTPLQAEYE
YVKYYPNGrsklVVNTPFVAVFSNFDSSQWEKADWANGSVFNCVWKP
SQVTFSNGKMILTLDREYvd*HHHHHHKDEL* 3' (SEQ ID NO:73).

In SEQ ID NO:73, the **bold/underlined** portion corresponds to the signal sequence, the *italicized/underlined* portion corresponds to the 6X His tag and endoplasmic reticulum retention sequence, and the two portions in lowercase letters correspond to restriction sites.

[059] Thus, any relevant nucleic acid encoding a HA polypeptide(s), fusion protein(s), or immunogenic portions thereof is intended to be encompassed within nucleic acid constructs provided herein.

[060] For production in plant systems, transgenic plants expressing HA polypeptide(s) (*e.g.*, HA polypeptide(s), fusion(s) thereof, and/or immunogenic portion(s) thereof) may be utilized. Alternatively or additionally, transgenic plants may be produced using methods well known in the art to generate stable production crops. Additionally, plants utilizing transient expression systems may be utilized for production of HA polypeptide(s). When utilizing plant expression systems, whether transgenic or transient expression in plants is utilized, any of nuclear expression, chloroplast expression, mitochondrial expression, or viral expression may be taken advantage of according to the applicability of the system to antigen desired. Furthermore, additional expression systems for production of antigens and fusion proteins can be utilized. For example, mammalian expression systems (*e.g.*, mammalian cell lines

such as CHO cells), bacterial expression systems (*e.g.*, *E. coli*), insect expression systems (*e.g.*, baculovirus), yeast expression systems, and *in vitro* expression systems (*e.g.*, reticulate lysates) may be used for expression of antigens and fusion proteins.

Production of Influenza Antigens

[061] Influenza antigens (including influenza protein(s), fragments, variants, and/or fusions thereof) can be produced in any suitable system; production is not limited to plant systems. Vector constructs and expression systems are well known in the art and may be adapted to incorporate use of influenza antigens provided herein. For example, influenza antigens (including fragments, variants, and/or fusions) can be produced in known expression systems, including mammalian cell systems, transgenic animals, microbial expression systems, insect cell systems, and plant systems, including transgenic and transient plant systems. Particularly where influenza antigens are produced as fusion proteins, it may be desirable to produce such fusion proteins in plant systems.

[062] In some embodiments, influenza antigens are produced in plant systems. Plants are relatively easy to manipulate genetically, and have several advantages over alternative sources such as human fluids, animal cell lines, recombinant microorganisms and transgenic animals. Plants have sophisticated post-translational modification machinery for proteins that is similar to that of mammals (although it should be noted that there are some differences in glycosylation patterns between plants and mammals). This enables production of bioactive reagents in plant tissues. Also, plants can economically produce very large amounts of biomass without requiring sophisticated facilities. Moreover, plants are not subject to contamination with animal pathogens. Like liposomes and microcapsules, plant cells are expected to provide protection for passage of antigen to the gastrointestinal tract.

[063] Plants may be utilized for production of heterologous proteins via use of various production systems. One such system includes use of transgenic/genetically-modified plants where a gene encoding target product is permanently incorporated into the genome of the plant. Transgenic systems may generate crop production systems. A variety of foreign proteins, including many of mammalian origin and many vaccine candidate antigens, have been expressed in transgenic plants and shown to have functional activity (Tacket *et al.*, 2000, *J. Infect. Dis.*, 182:302; and Thanavala *et al.*, 2005, *Proc. Natl. Acad. Sci., USA*, 102:3378). Additionally, administration of unprocessed transgenic plants expressing hepatitis B major surface antigen to non-immunized human volunteers resulted in production of immune response (Kapusta *et al.*, 1999, *FASEB J.*, 13:1796).

[064] Another system for expressing polypeptides in plants utilizes plant viral vectors engineered to express foreign sequences (*e.g.*, transient expression). This approach allows for use of healthy non-transgenic plants as rapid production systems. Thus, genetically engineered plants and plants infected with recombinant plant viruses can serve as "green factories" to rapidly generate and produce specific proteins of interest. Plant viruses have certain advantages that make them attractive as expression vectors for foreign protein production. Several members of plant RNA viruses have been well characterized, and infectious cDNA clones are available to facilitate genetic manipulation. Once infectious viral genetic material enters a susceptible host cell, it replicates to high levels and spreads rapidly throughout the entire plant. There are several approaches to producing target polypeptides using plant viral expression vectors, including incorporation of target polypeptides into viral genomes. One approach involves engineering coat proteins of viruses that infect bacteria, animals or plants to function as carrier molecules for antigenic peptides. Such carrier proteins have the potential to assemble and form recombinant virus-like particles displaying desired antigenic epitopes on their surface. This approach allows for time-efficient production of antigen and/or antibody candidates, since the particulate nature of an antigen and/or antibody candidate facilitates easy and cost-effective recovery from plant tissue. Additional advantages include enhanced target-specific immunogenicity, the potential to incorporate multiple antigen determinants and/or antibody sequences, and ease of formulation into antigen and/or antibody that can be delivered nasally, orally or parenterally. As an example, spinach leaves containing recombinant plant viral particles carrying epitopes of virus fused to coat protein have generated immune response upon administration (Modelska *et al.*, 1998, *Proc. Natl. Acad. Sci., USA*, 95:2481; and Yusibov *et al.*, 2002, *Vaccine*, 19/20:3155).

Production of Hemagglutinin Antigens

[065] HA antigens (including HA polypeptide(s), fusions thereof, and/or immunogenic portions thereof) may be produced in any suitable system; production is not limited to plant systems. Vector constructs and expression systems are well known in the art and may be adapted to incorporate use of HA polypeptides provided herein. For example, HA polypeptides can be produced in known expression systems, including mammalian cell systems, transgenic animals, microbial expression systems, insect cell systems, and plant systems, including transgenic and transient plant systems. Particularly where HA

polypeptides are produced as fusion proteins, it may be desirable to produce such fusion proteins in plant systems.

[066] In some embodiments, HA polypeptides are desirably produced in plant systems. Plants are relatively easy to manipulate genetically, and have several advantages over alternative sources such as human fluids, animal cell lines, recombinant microorganisms and transgenic animals. Plants have sophisticated post-translational modification machinery for proteins that is similar to that of mammals (although it should be noted that there are some differences in glycosylation patterns between plants and mammals). This enables production of bioactive reagents in plant tissues. Also, plants can economically produce very large amounts of biomass without requiring sophisticated facilities. Moreover, plants are not subject to contamination with animal pathogens. Like liposomes and microcapsules, plant cells are expected to provide protection for passage of antigen to the gastrointestinal tract.

[067] Plants may be utilized for production of heterologous proteins via use of various production systems. One such system includes use of transgenic/genetically-modified plants where a gene encoding target product is permanently incorporated into the genome of the plant. Transgenic systems may generate crop production systems. A variety of foreign proteins, including many of mammalian origin and many vaccine candidate antigens, have been expressed in transgenic plants and shown to have functional activity. (Tacket *et al.*, 2000, *J. Infect. Dis.*, 182:302; and Thanavala *et al.*, 2005, *Proc. Natl. Acad. Sci., USA*, 102:3378). Additionally, administration
of unprocessed transgenic plants expressing hepatitis B major surface antigen to non-immunized human volunteers resulted in production of immune response (Kapusta *et al.*, 1999, *FASEB J.*, 13:1796).

[068] One system for expressing polypeptides in plants utilizes plant viral vectors engineered to express foreign sequences (*e.g.*, transient expression). This approach allows for use of healthy non-transgenic plants as rapid production systems. Thus, genetically engineered plants and plants infected with recombinant plant viruses can serve as "green factories" to rapidly generate and produce specific proteins of interest. Plant viruses have certain advantages that make them attractive as expression vectors for foreign protein production. Several members of plant RNA viruses have been well characterized, and infectious cDNA clones are available to facilitate genetic manipulation. Once infectious viral genetic material enters a susceptible host cell, it replicates to high levels and spreads rapidly throughout the entire plant. There are several approaches to producing target polypeptides using plant viral expression vectors, including incorporation of target polypeptides into viral

genomes. One approach involves engineering coat proteins of viruses that infect bacteria, animals or plants to function as carrier molecules for antigenic peptides. Such carrier proteins have the potential to assemble and form recombinant virus-like particles displaying desired antigenic epitopes on their surface. This approach allows for time-efficient production of vaccine candidates, since the particulate nature of a vaccine candidate facilitates easy and cost-effective recovery from plant tissue. Additional advantages include enhanced target-specific immunogenicity, the potential to incorporate multiple vaccine determinants, and ease of formulation into vaccines that can be delivered nasally, orally or parenterally. As an example, spinach leaves containing recombinant plant viral particles carrying epitopes of virus fused to coat protein have generated immune response upon administration (Modelska *et al.*, 1998, *Proc. Natl. Acad. Sci., USA*, 95:2481; and Yusibov *et al.*, 2002, *Vaccine*, 19/20:3155).

Plant Expression Systems

[069] The teachings herein are applicable to a wide variety of different plants. In general, any plants that are amendable to expression of introduced constructs as described herein are useful in accordance with the methods disclosed herein. In some embodiments, it is desirable to use young plants in order to improve the speed of protein/polypeptide production. As indicated here, in many embodiments, sprouted seedlings are utilized. As is known in the art, most sprouts are quick growing, edible plants produced from storage seeds. However, those of ordinary skill in the art will appreciate that the term "sprouted seedling" has been used herein in a more general context, to refer to young plants whether or not of a variety typically classified as "sprouts." Any plant that is grown long enough to have sufficient green biomass to allow introduction and/or expression of an expression construct as provided for herein (recognizing that the relevant time may vary depending on the mode of delivery and/or expression of the expression construct) can be considered a "sprouted seedling" herein.

[070] In many embodiments, edible plants are utilized (*i.e.*, plants that are edible by – not toxic to – the subject to whom the protein or polypeptide is to be administered).

[071] Any plant susceptible to incorporation and/or maintenance of heterologous nucleic acid and capable of producing heterologous protein can be utilized. In general, it may be desirable to utilize plants that are amenable to growth under defined conditions, for example in a greenhouse and/or in aqueous systems. It may be desirable to select plants that are not typically consumed by human beings or domesticated animals and/or are not typically part of the human food chain, so that they may be grown outside without concern that expressed polynucleotide may be undesirably ingested. In some embodiments, however, it will be

desirable to employ edible plants. In particular embodiments, it will be desirable to utilize plants that accumulate expressed polypeptides in edible portions of a plant.

[072] Often, certain desirable plant characteristics will be determined by the particular polynucleotide to be expressed. To give but a few examples, when a polynucleotide encodes a protein to be produced in high yield (as will often be the case, for example, when antigen proteins are to be expressed), it will often be desirable to select plants with relatively high biomass (*e.g.*, tobacco, which has additional advantages that it is highly susceptible to viral infection, has a short growth period, and is not in the human food chain). Where a polynucleotide encodes antigen protein whose full activity requires (or is inhibited by) a particular post-translational modification, the ability (or inability) of certain plant species to accomplish relevant modification (*e.g.*, a particular glycosylation) may direct selection. For example, plants are capable of accomplishing certain post-translational modifications (*e.g.*, glycosylation), however, plants will not generate sialyation patterns which are found in mammalian post-translational modification. Thus, plant production of antigen may result in production of a different entity than the identical protein sequence produced in alternative systems.

[073] In certain embodiments, crop plants, or crop-related plants are utilized. In certain specific embodiments, edible plants are utilized.

[074] Plants for use in accordance with the methods provided herein include, for example, Angiosperms, Bryophytes (*e.g.*, Hepaticae and Musci), Pteridophytes (*e.g.*, ferns, horsetails, and lycopods), Gymnosperms (*e.g.*, conifers, cycase, Ginko, and Gnetales), and Algae (*e.g.*, Chlorophyceae, Phaeophyceae, Rhodophyceae, Myxophyceae, Xanthophyceae, and Euglenophyceae). Exemplary plants include members of the families Leguminosae (Fabaceae; *e.g.*, pea, alfalfa, and soybean); Gramineae (Poaceae; *e.g.*, corn, wheat, and rice); Solanaceae, particularly of the genus *Lycopersicon* (*e.g.*, tomato), *Solanum* (*e.g.*, potato and eggplant), *Capsium* (*e.g.*, pepper), *Nicotiana* (*e.g.*, tobacco); Umbelliferae, particularly of the genus *Daucus* (*e.g.*, carrot), *Apium* (*e.g.*, celery), or *Rutaceae* (*e.g.*, oranges); Compositae, particularly of the genus *Lactuca* (*e.g.*, lettuce); and Brassicaceae (Cruciferae), particularly of the genus *Brassica* or *Sinapis*. In certain aspects, useful plants may be species of *Brassica* or *Arabidopsis*. Some exemplary Brassicaceae family members include *Brassica campestris, B. carinata, B. juncea, B. napus, B. nigra, B. oleraceae, B. tournifortii, Sinapis alba,* and *Raphanus sativus.* Some suitable plants that are amendable to transformation and are edible as sprouted seedlings include alfalfa, mung bean, radish, wheat, mustard, spinach, carrot, beet, onion, garlic, celery, rhubarb, a leafy plant such as cabbage or lettuce, watercress or

cress, herbs such as parsley, mint, or clovers, cauliflower, broccoli, soybean, lentils, and edible flowers such as sunflower.

[075] A wide variety of plant species may be suitable in the practices described herein. For example, a variety of different bean and other species including, for example, adzuki bean, alfalfa, barley, broccoli, bill jump pea, buckwheat, cabbage, cauliflower, clover, collard greens, fenugreek, flax, garbanzo bean, green pea, Japanese spinach, kale, kamut, kohlrabi, marrowfat pea, mung bean, mustard greens, pinto bean, radish, red clover, soy bean, speckled pea, sunflower, turnip, yellow trapper pea, and others may be amenable to the production of heterologous proteins from viral vectors launched from an agrobacterial construct (*e.g.*, introduced by agroinfiltration). In some embodiments, bill jump pea, green pea, marrowfat pea, speckled pea, and/or yellow trapper pea are particularly useful. In certain embodiments, therefore, this document provides production of proteins or polypeptides (*e.g.*, antigens) in one or more of these plants using an agrobacterial vector that launches a viral construct (*i.e.*, an RNA with characteristics of a plant virus) encoding the relevant protein or polypeptide of interest. In some embodiments, the RNA has characteristics of (and/or includes sequences of) AlMV. In some embodiments, the RNA has characteristics of (and/or includes sequences of) TMV.

[076] It will be appreciated that, in one aspect, this document provides young plants (*e.g.*, sprouted seedlings) that express a target protein or polypeptide of interest. In some embodiments, the young plants were grown from transgenic seeds; this document also provides seeds which can be generated and/or utilized for the methods described herein. Seeds transgenic for any gene of interest can be sprouted and optionally induced for production of a protein or polypeptide of interest. For example, seeds capable of expressing any gene of interest can be sprouted and induced through: i) virus infection, ii) agroinfiltration, or iii) bacteria that contain virus genome. Seeds capable of expressing a transgene for heavy or light chain of any monoclonal antibody can be sprouted and induced for production of full-length molecule through: i) virus infection, ii) agroinfiltration, or iii) inoculation with bacteria that contain virus genome. Seeds capable of expressing a transgene for one or more components of a complex molecule comprising multiple components such as sIgA can be sprouted and used for producing a fully functional molecule through: i) virus infection, ii) agroinfiltration, or iii) inoculation with bacteria that contain virus genome. Seeds from healthy non-transgenic plants can be sprouted and used for producing target sequences through: i) virus infection, ii) agroinfiltration, or iii) inoculation with bacteria that contain a virus genome.

[077] In some embodiments, the young plants were grown from seeds that were not transgenic. Typically, such young plants will harbor viral sequences that direct expression of the protein or polypeptide of interest. In some embodiments, the plants may also harbor agrobacterial sequences, optionally including sequences that "launched" the viral sequences.

Introducing Vectors into Plants

[078] In general, vectors may be delivered to plants according to known techniques. For example, vectors themselves may be directly applied to plants (*e.g.*, via abrasive inoculations, mechanized spray inoculations, vacuum infiltration, particle bombardment, or electroporation). Alternatively or additionally, virions may be prepared (*e.g.*, from already infected plants), and may be applied to other plants according to known techniques.

[079] A wide variety of viruses are known that infect various plant species, and can be employed for polynucleotide expression (see, for example, in *The Classification and Nomenclature of Viruses*, "Sixth Report of the International Committee on Taxonomy of Viruses" (Ed. Murphy *et al.*), Springer Verlag: New York, 1995; Grierson *et al.*, *Plant Molecular Biology*, Blackie, London, pp. 126-146, 1984; Gluzman *et al.*, *Communications in Molecular Biology: Viral Vectors*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 172-189, 1988; and Mathew, *Plant Viruses Online*).

In certain embodiments, rather than delivering a single viral vector to a plant cell, multiple different vectors are delivered which, together, allow for replication (and, optionally cell-to-cell and/or long distance movement) of viral vector(s). Some or all of the proteins may be encoded by the genome of transgenic plants. In certain aspects, described in further detail herein, these systems include one or more viral vector components.

[080] Vector systems that include components of two heterologous plant viruses in order to achieve a system that readily infects a wide range of plant types and yet poses little or no risk of infectious spread. An exemplary system has been described previously (see, *e.g.*, PCT Publication WO 00/25574 and U.S. Patent Publication 2005/0026291).

As noted herein, viral vectors can be applied to plants (*e.g.*, plants, portions of plant, or sprouts), through infiltration, mechanical inoculation, or spraying, for example. Where infection is to be accomplished by direct application of a viral genome to a plant, any available technique may be used to prepare the genome. For example, many viruses that are usefully employed in accordance with the present disclosure have ssRNA genomes. ssRNA may be prepared by transcription of a DNA copy of the genome, or by replication of an RNA copy, either *in vivo* or *in vitro*. Given the readily availability of

easy-to-use *in vitro* transcription systems (*e.g.*, SP6, T7, and reticulocyte lysate), and also the convenience of maintaining a DNA copy of an RNA vector, ssRNA vectors may be prepared by *in vitro* transcription, particularly with T7 or SP6 polymerase.

[081] In certain embodiments, rather than introducing a single viral vector type into a plant, multiple different viral vectors are introduced. Such vectors may, for example, trans-complement each other with respect to functions such as replication, cell-to-cell movement, and/or long distance movement. Vectors may contain different polynucleotides encoding HA polypeptides as provided herein. Selection for plant(s) or portions thereof that express multiple polypeptides encoding one or more HA polypeptide(s) may be performed as described above for single polynucleotides or polypeptides.

Plant Tissue Expression Systems

[082] As discussed herein, HA polypeptides may be produced in any desirable system. Vector constructs and expression systems are well known in the art and may be adapted to incorporate use of HA polypeptides provided herein. For example, transgenic plant production is known and generation of constructs and plant production may be adapted according to known techniques in the art. In some embodiments, transient expression systems in plants are desirable. Two of these systems include production of clonal roots and clonal plant systems, and derivatives thereof, as well as production of sprouted seedlings systems.

Clonal Plants

[083] Clonal roots maintain RNA viral expression vectors and stably produce target protein uniformly in an entire root over extended periods of time and multiple subcultures. In contrast to plants, where a target gene is eliminated via recombination during cell-to-cell or long distance movement, in root cultures the integrity of a viral vector is maintained and levels of target protein produced over time are similar to those observed during initial screening. Clonal roots allow for ease of production of heterologous protein material for oral formulation of antigen and vaccine compositions. Methods and reagents for generating a variety of clonal entities derived from plants which are useful for production of antigen (*e.g.*, antigen proteins as provided herein) have been described previously and are known in the art (see, for example, PCT Publication WO 05/81905). Clonal entities include clonal root lines, clonal root cell lines, clonal plant cell lines, and clonal plants capable of production of antigen (*e.g.*, antigen proteins as described herein). This document further provides methods and reagents for expression of antigen polynucleotide

and polypeptide products in clonal cell lines derived from various plant tissues (*e.g.*, roots, leaves), and in whole plants derived from single cells (clonal plants). Such methods are typically based on use of plant viral vectors of various types.

[084] For example, in one aspect, this document provides methods of obtaining a clonal root line that expresses a polynucleotide encoding a HA polypeptide, comprising the steps of: (i) introducing a viral vector that comprises a polynucleotide encoding an HA polypeptide as described herein into a plant or portion thereof; and (ii) generating one or more clonal root lines from a plant. Clonal root lines may be generated, for example, by infecting a plant or plant portion (*e.g.*, a harvested piece of leaf) with an *Agrobacterium* (*e.g.*, *A. rhizogenes*) that causes formation of hairy roots. Clonal root lines can be screened in various ways to identify lines that maintain virus, or lines that express a polynucleotide encoding a HA polypeptide at high levels, for example. This document further provides clonal root lines, *e.g.*, clonal root lines produced as described herein, and further encompasses methods of expressing polynucleotides and producing polypeptide(s) encoding HA polypeptide(s) using clonal root lines.

[085] This document further provides methods of generating a clonal root cell line that expresses a polynucleotide encoding a HA polypeptide, comprising the steps of: (i) generating a clonal root line, cells of which contain a viral vector whose genome comprises a polynucleotide encoding a HA polypeptide; (ii) releasing individual cells from a clonal root line; and (iii) maintaining cells under conditions suitable for root cell proliferation. Clonal root cell lines and methods of expressing polynucleotides and producing polypeptides using clonal root cell lines also are provided herein.

[086] In some aspects, this document provides methods of generating a clonal plant cell line that expresses a polynucleotide encoding a HA polypeptide, comprising the steps of: (i) generating a clonal root line, cells of which contain a viral vector whose genome comprises a polynucleotide encoding a HA polypeptide; (ii) releasing individual cells from a clonal root line; and (iii) maintaining cells in culture under conditions appropriate for plant cell proliferation. Also provided herein are methods for generating a clonal plant cell line that expresses a polynucleotide encoding a HA polypeptide, comprising the steps of: (i) introducing into cells of a plant cell line maintained in culture a viral vector that comprises a polynucleotide encoding a HA polypeptide; and (ii) enriching for cells that contain viral vector. Enrichment may be performed, for example, by (i) removing a portion of cells from the culture; (ii) diluting removed cells so as to reduce cell concentration; (iii) allowing diluted

cells to proliferate; and (iv) screening for cells that contain viral vector. Clonal plant cell lines may be used for production of a HA polypeptide as provided herein.

[087] This document provides a number of methods for generating clonal plants, cells of which contain a viral vector that comprises a polynucleotide encoding a HA polypeptide as disclosed herein. For example, this document provides methods of generating a clonal plant that expresses a polynucleotide encoding a HA polypeptide, comprising the steps of: (i) generating a clonal root line, cells of which contain a viral vector whose genome comprises a polynucleotide encoding a HA polypeptide; (ii) releasing individual cells from a clonal root line; and (iii) maintaining released cells under conditions appropriate for formation of a plant. This document further provides methods for generating a clonal plant that expresses a polynucleotide encoding a HA polypeptide, comprising the steps of: (i) generating a clonal plant cell line, cells of which contain a viral vector whose genome comprises a polynucleotide encoding a HA polypeptide; and (ii) maintaining cells under conditions appropriate for formation of a plant. In general, clonal plants as provided herein can express any polynucleotide encoding a HA polypeptide in accordance with this document. Such clonal plants can be used for production of an antigen polypeptide.

[088] As noted above, this document provides systems for expressing a polynucleotide or polynucleotide(s) encoding HA polypeptide(s) in clonal root lines, clonal root cell lines, clonal plant cell lines (*e.g.*, cell lines derived from leaf or stem), and in clonal plants. A polynucleotide encoding a HA polypeptide can be introduced into an ancestral plant cell using a plant viral vector whose genome includes polynucleotide encoding an HA polypeptide operably linked to (*i.e.*, under control of) a promoter. A clonal root line or clonal plant cell line can established from a cell containing virus according to any of several techniques, including those that are further described below. The plant virus vector or portions thereof can be introduced into a plant cell by infection, by inoculation with a viral transcript or infectious cDNA clone, by electroporation, or by T-DNA mediated gene transfer, for example.

[089] The following sections describe methods for generating clonal root lines, clonal root cell lines, clonal plant cell lines, and clonal plants that express a polynucleotide encoding a HA polypeptide as provided herein. A "root line" is distinguished from a "root cell line" in that a root line produces actual rootlike structures or roots while a root cell line consists of root cells that do not form rootlike structures. Use of the term "line" is intended to indicate that cells of the line can proliferate and pass genetic information on to progeny cells. Cells of a cell line typically proliferate in culture without being part of an organized structure such as

those found in an intact plant. Use of the term "root line" is intended to indicate that cells in the root structure can proliferate without being part of a complete plant. It is noted that the term "plant cell" encompasses root cells. However, to distinguish methods for generating root lines and root cell lines from those used to directly generate plant cell lines from non-root tissue (as opposed to generating clonal plant cell lines from clonal root lines or clonal plants derived from clonal root lines), the terms "plant cell" and "plant cell line" as used herein generally refer to cells and cell lines that consist of non-root plant tissue. Plant cells can be from, for example, leaf, stem, shoot, or flower part. It is noted that seeds can be derived from clonal plants generated as derived herein. Such seeds may contain viral vector as will plants obtained from such seeds. Methods for obtaining seed stocks are well known in the art (see, for example, U.S. Patent Publication 2004/093643).

Clonal Root Lines

[090] This document provides systems for generating a clonal root line in which a plant viral vector is used to direct expression of a polynucleotide encoding a HA polypeptide. One or more viral expression vector(s) including a polynucleotide encoding a HA polypeptide operably linked to a promoter can be introduced into a plant or a portion thereof according to any of a variety of known methods. For example, plant leaves can be inoculated with viral transcripts. Vectors themselves may be directly applied to plants (*e.g.*, via abrasive inoculations, mechanized spray inoculations, vacuum infiltration, particle bombardment, or electroporation). Alternatively or additionally, virions may be prepared (*e.g.*, from already infected plants), and may be applied to other plants according to known techniques.

[091] Where infection is to be accomplished by direct application of a viral genome to a plant, any available technique may be used to prepare viral genome. For example, many viruses that are usefully employed in accordance with the present disclosure have ssRNA genomes. ssRNA may be prepared by transcription of a DNA copy of the genome, or by replication of an RNA copy, either *in vivo* or *in vitro*. Given the readily available, easy-to-use *in vitro* transcription systems (*e.g.*, SP6, T7, and reticulocyte lysate), and also the convenience of maintaining a DNA copy of an RNA vector, ssRNA vectors can be prepared by *in vitro* transcription, particularly with T7 or SP6 polymerase. Infectious cDNA clones can be used. *Agrobacterially* mediated gene transfer can be used to transfer viral nucleic acids such as viral vectors (either entire viral genomes or portions thereof) to plant cells using, *e.g.*, agroinfiltration, according to methods known in the art.

[092] A plant or plant portion may then be then maintained (*e.g.*, cultured or grown) under conditions suitable for replication of viral transcript. In certain embodiments, virus spreads beyond the initially inoculated cell, *e.g.*, locally from cell to cell and/or systemically from an initially inoculated leaf into additional leaves. However, in some embodiments, virus does not spread. Thus viral vector may contain genes encoding functional MP and/or CP, but may be lacking one or both of such genes. In general, viral vector is introduced into (infects) multiple cells in the plant or portion thereof.

[093] Following introduction of viral vector into a plant, leaves are harvested. In general, leaves may be harvested at any time following introduction of a viral vector. However, it may be desirable to maintain a plant for a period of time following introduction of a viral vector into the plant, *e.g.*, a period of time sufficient for viral replication and, optionally, spread of virus from the cells into which it was initially introduced. A clonal root culture (or multiple cultures) is prepared, *e.g.*, by known methods further described below.

[094] In general, any available method may be used to prepare a clonal root culture from a plant or plant tissue into which a viral vector has been introduced. One such method employs genes that exist in certain bacterial plasmids. These plasmids are found in various species of *Agrobacterium* that infect and transfer DNA to a wide variety of organisms. As a genus, *Agrobacteria* can transfer DNA to a large and diverse set of plant types including numerous dicot and monocot angiosperm species and gymnosperms (see, for example, Gelvin, 2003, *Microbiol. Mol. Biol. Rev.*, 67:16, and references therein).

The molecular basis of genetic transformation of plant cells is transfer from bacterium and integration into plant nuclear genome of a region of a large tumor-inducing (Ti) or rhizogenic (Ri) plasmid that resides within various *Agrobacterial* species. This region is referred to as the T-region when present in the plasmid and as T-DNA when excised from plasmid. Generally, a single-stranded T-DNA molecule is transferred to a plant cell in naturally occurring *Agrobacterial* infection and is ultimately incorporated (in double-stranded form) into the genome. Systems based on Ti plasmids are widely used for introduction of foreign genetic material into plants and for production of transgenic plants.

[095] Infection of plants with various *Agrobacterial* species and transfer of T-DNA has a number of effects. For example, *A. tumefaciens* causes crown gall disease while *A. rhizogenes* causes development of hairy roots at the site of infection, a condition known as "hairy root disease." Each root arises from a single genetically transformed cell. Thus root cells in roots are clonal, and each root represents a clonal population of cells. Roots produced by *A. rhizogenes* infection are characterized by a high growth rate and genetic stability (Giri

et al., 2000, *Biotech. Adv.*, 18:1, and references therein). In addition, such roots are able to regenerate genetically stable plants (Giri 2000, *supra*).

[096] In general, this document encompasses use of any strain of *Agrobacteria*, particularly any *A. rhizogenes* strain, that is capable of inducing formation of roots from plant cells. As mentioned above, a portion of the Ri plasmid (Ri T-DNA) is responsible for causing hairy root disease. While transfer of this portion of the Ri plasmid to plant cells can conveniently be accomplished by infection with *Agrobacteria* harboring the Ri plasmid, this document encompasses use of alternative methods of introducing the relevant region into a plant cell. Such methods include any available method of introducing genetic material into plant cells including, but not limited to, biolistics, electroporation, PEG-mediated DNA uptake, and Ti-based vectors. The relevant portions of Ri T-DNA can be introduced into plant cells by use of a viral vector. Ri genes can be included in the same vector that contains a polynucleotide encoding a HA polypeptide or in a different viral vector, which can be the same or a different type to that of the vector that contains a polynucleotide encoding a HA polypeptide as provided herein. It is noted that the entire Ri T-DNA may not be required for production of hairy roots, and this document encompasses use of portions of Ri T-DNA, provided that such portions contain sufficient genetic material to induce root formation, as known in the art. Additional genetic material, *e.g.*, genes present within the Ri plasmid but not within T-DNA, may be transferred to a plant cell, particularly genes whose expression products facilitate integration of T-DNA into the plant cell DNA.

[097] In order to prepare a clonal root line in accordance with certain embodiments, harvested leaf portions are contacted with *A. rhizogenes* under conditions suitable for infection and transformation. Leaf portions are maintained in culture to allow development of hairy roots. Each root is clonal, *i.e.*, cells in the root are derived from a single ancestral cell into which Ri T-DNA was transferred. In some embodiments, a portion of such ancestral cells will contain a viral vector. Thus cells in a root derived from such an ancestral cell may contain viral vector since it will be replicated and will be transmitted during cell division. Thus a high proportion (*e.g.*, at least 50%, at least 75%, at least 80%, at least 90%, at least 95%), all (100%), or substantially all (at least 98%) of cells will contain viral vector. It is noted that since viral vector is inherited by daughter cells within the clonal root, movement of viral vector within the root is not necessary to maintain viral vector throughout the root. Individual clonal hairy roots may be removed from the leaf portion and further cultured.

Such roots are also referred to herein as root lines. Isolated clonal roots continue to grow following isolation.

[098] A variety of different clonal root lines have been generated using methods as described herein. These root lines were generated using viral vectors containing polynucleotide(s) encoding a HA polypeptide as provided herein (*e.g.*, encoding HA polypeptide(s), fusions thereof, and/or immunogenic portions thereof). Root lines were tested by Western blot. Root lines displayed a variety of different expression levels of various polypeptides. Root lines displaying high expression were selected and further cultured. These root lines were subsequently tested again and shown to maintain high levels of expression over extended periods of time, indicating stability. Expression levels were comparable to or greater than expression in intact plants infected with the same viral vector used to generate clonal root lines. In addition, stability of expression of root lines was superior to that obtained in plants infected with the same viral vector. Up to 80% of such virus-infected plants reverted to wild type after 2 – 3 passages. (Such passages involved inoculating plants with transcripts, allowing infection (local or systemic) to become established, taking a leaf sample, and inoculating fresh plants that are subsequently tested for expression).

[099] Root lines may be cultured on a large scale for production of antigen polypeptides, as discussed further below. It is noted that clonal root lines (and cell lines derived from clonal root lines) can generally be maintained in medium that does not include various compounds, *e.g.*, plant growth hormones such as auxins and cytokinins, that typically are employed in culture of root and plant cells. This feature greatly reduces expense associated with tissue culture, and it may contribute significantly to economic feasibility of protein production using plants.

[0100] Any of a variety of methods may be used to select clonal roots that express a polynucleotide encoding HA polypeptide(s) as provided herein. Western blots, ELISA assays, and other suitable techniques can be used to detect an encoded polypeptide. In the case of detectable markers such as GFP, alternative methods such as visual screens can be performed. If a viral vector that contains a polynucleotide that encodes a selectable marker is used, an appropriate selection can be imposed (*e.g.*, leaf material and/or roots derived therefrom can be cultured in the presence of an appropriate antibiotic or nutritional condition and surviving roots identified and isolated). Certain viral vectors contain two or more polynucleotide(s) encoding HA polypeptide(s), *e.g.*, two or more polynucleotides encoding different polypeptides. If one of these is a selectable or detectable marker, clonal roots that

are selected or detected by selecting for or detecting expression of the marker will have a high probability of also expressing a second polynucleotide. Screening for root lines that contain particular polynucleotides can also be performed using PCR and other nucleic acid detection methods.

[0101] Alternatively or additionally, clonal root lines can be screened for presence of virus by inoculating host plants that will form local lesions as a result of virus infection (*e.g.*, hypersensitive host plants). For example, 5 mg of root tissue can be homogenized in 50 µl of phosphate buffer and used to inoculate a single leaf of a tobacco plant. If virus is present in root cultures, within two to three days characteristic lesions will appear on infected leaves. This means that root line contains recombinant virus that carries a polynucleotide encoding a HA polypeptide. If no local lesions are formed, there is no virus, and the root line is rejected as negative. This method is highly time and cost efficient. After initially screening for the presence of virus, roots that contain virus may be subjected to secondary screening, *e.g.*, by Western blot or ELISA to select high expressers. Additional screens can be applied, such as screens for rapid growth, growth in particular media, or growth under particular environmental conditions, for example. These screening methods may, in general, be applied in the development of any of clonal root lines, clonal root cell lines, clonal plant cell lines, and/or clonal plants described herein.

[0102] As will be evident to one of ordinary skill in the art, a variety of modifications may be made to the methods for generating clonal root lines that contain a viral vector, and such modifications are within the scope of this document. For example, while it is generally desirable to introduce viral vector into an intact plant or portion thereof prior to introduction of Ri T-DNA genes, in certain embodiments, the Ri-DNA is introduced prior to introducing viral vector. In addition, it is possible to contact intact plants with *A. rhizogenes* rather than harvesting leaf portions and then exposing them to bacterium.

[0103] Other methods of generating clonal root lines from single cells of a plant or portion thereof that harbor a viral vector can be used (*i.e.*, methods not using *A. rhizogenes* or genetic material from the Ri plasmid). For example, treatment with certain plant hormones or combinations of plant hormones is known to result in generation of roots from plant tissue.

Clonal Cell Lines Derived from Clonal Root Lines

[0104] As described above, this document provides methods for generating clonal root lines, wherein cells in root lines contain a viral vector. As is well known in the art, a variety of different cell lines can be generated from roots. For example, root cell lines can be generated from individual root cells obtained from a root using a variety of known methods. Such root

cell lines may be obtained from various different root cell types within the root. In general, root material is harvested and dissociated (*e.g.*, physically and/or enzymatically digested) to release individual root cells, which are then further cultured. Complete protoplast formation is generally not necessary. If desired, root cells can be plated at very dilute cell concentrations, so as to obtain root cell lines from single root cells. Root cell lines derived in this manner are clonal root cell lines containing viral vector. Such root cell lines therefore exhibit stable expression of a polynucleotide encoding a HA polypeptide as provided herein. Clonal plant cell lines can be obtained in a similar manner from clonal roots, *e.g.*, by culturing dissociated root cells in the presence of appropriate plant hormones. Screens and successive rounds of enrichment can be used to identify cell lines that express a polynucleotide encoding a HA polypeptide at high levels. However, if the clonal root line from which the cell line is derived already expresses at high levels, such additional screens may be unnecessary.

[0105] As in the case of the clonal root lines, cells of a clonal root cell line are derived from a single ancestral cell that contains viral vector and will, therefore, also contain viral vector since it will be replicated and will be transmitted during cell division. Thus a high proportion (*e.g.* at least 50%, at least 75%, at least 80%, at least 90%, at least 95%), all (100%), or substantially all (at least 98%) of cells will contain viral vector. It is noted that since viral vector is inherited by daughter cells within a clonal root cell line, movement of viral vector among cells is not necessary to maintain viral vector. Clonal root cell lines can be used for production of a polynucleotide encoding a HA polypeptide, as described below.

Clonal Plant Cell Lines

[0106] This document provides methods for generating a clonal plant cell line in which a plant viral vector is used to direct expression of a polynucleotide encoding a HA polypeptide as provided herein. According to these methods, one or more viral expression vector(s) including a polynucleotide encoding a HA polypeptide operably linked to a promoter is introduced into cells of a plant cell line that is maintained in cell culture. A number of plant cell lines from various plant types are known in the art, any of which can be used. Newly derived cell lines can be generated according to known methods for use in practicing the methods disclosed herein. A viral vector can be introduced into cells of a plant cell line according to any of a number of methods. For example, protoplasts can be made and viral transcripts then electroporated into cells. Other methods of introducing a plant viral vector into cells of a plant cell line also can be used.

[0107] A method for generating clonal plant cell lines and a viral vector suitable for introduction into plant cells (*e.g.*, protoplasts) can be used as follows: Following introduction of viral vector, a plant cell line may be maintained in tissue culture. During this time viral vector may replicate, and polynucleotide(s) encoding a HA polypeptide(s) as provided herein may be expressed. Clonal plant cell lines are derived from culture, *e.g.*, by a process of successive enrichment. For example, samples may be removed from culture, optionally with dilution so that the concentration of cells is low, and plated in Petri dishes in individual droplets. Droplets are then maintained to allow cell division.

[0108] It will be appreciated that droplets may contain a variable number of cells, depending on the initial density of the culture and the amount of dilution. Cells can be diluted such that most droplets contain either 0 or 1 cell if it is desired to obtain clonal cell lines expressing a polynucleotide encoding a HA polypeptide after only a single round of enrichment. However, it can be more efficient to select a concentration such that multiple cells are present in each droplet and then screen droplets to identify those that contain expressing cells. In general, any appropriate screening procedure can be employed. For example, selection or detection of a detectable marker such as GFP can be used. Western blots or ELISA assays can be used. Individual droplets (100 μl) contain more than enough cells for performance of these assays. Multiple rounds of enrichment are performed to isolate successively higher expressing cell lines. Single clonal plant cell lines (*i.e.*, populations derived from a single ancestral cell) can be generated by further limiting dilution using standard methods for single cell cloning. However, it is not necessary to isolate individual clonal lines. A population containing multiple clonal cell lines can be used for expression of a polynucleotide encoding one or more HA polypeptide(s).

[0109] In general, certain considerations described above for generation of clonal root lines apply to the generation of clonal plant cell lines. For example, a diversity of viral vectors containing one or more polynucleotide(s) encoding a HA polypeptide(s) as provided herein can be used as can combinations of multiple different vectors. Similar screening methods can be used. As in the case of clonal root lines and clonal root cell lines, cells of a clonal plant cell line are derived from a single ancestral cell that contains viral vector and will, therefore, also contain viral vector since it will be replicated and will be transmitted during cell division. Thus a high proportion (*e.g.* at least 50%, at least 75%, at least 80%, at least 90%, at least 95%), all (100%), or substantially all (at least 98%) of cells will contain viral vector. It is noted that since viral vector is inherited by daughter cells within a clonal plant cell line, movement of viral vector among cells is not necessary to maintain viral vector. The clonal

plant cell line can be used for production of a polypeptide encoding a HA polypeptide as described below.

Clonal Plants

[0110] Clonal plants can be generated from clonal roots, clonal root cell lines, and/or clonal plant cell lines produced according to various methods described herein. Methods for generation of plants from roots, root cell lines, and plant cell lines such as clonal root lines, clonal root cell lines, and clonal plant cell lines described herein are well known in the art (see, *e.g.*, Peres *et al.*, 2001, *Plant Cell, Tissue, Organ Culture*, 65:37;
and standard reference works on plant molecular biology and biotechnology cited elsewhere herein). This document therefore provides a method of generating a clonal plant comprising steps of (i) generating a clonal root line, clonal root cell line, or clonal plant cell line according to any of the methods described herein; and (ii) generating a whole plant from a clonal root line, clonal root cell line, or clonal plant. Clonal plants may be propagated and grown according to standard methods.

[0111] As in the case of clonal root lines, clonal root cell lines, and clonal plant cell lines, cells of a clonal plant are derived from a single ancestral cell that contains viral vector and will, therefore, also contain viral vector since it will be replicated and will be transmitted during cell division. Thus a high proportion (*e.g.* at least 50%, at least 75%, at least 80%, at least 90%, at least 95%), all (100%), or substantially all (at least 98%) of cells will contain viral vector. It is noted that since viral vector is inherited by daughter cells within the clonal plant, movement of viral vector is not necessary to maintain viral vector.

Sprouts and Sprouted Seedling Plant Expression Systems

[0112] Any of a variety of different systems can be used to express proteins or polypeptides in young plants (*e.g.*, sprouted seedlings). In some embodiments, transgenic cell lines or seeds are generated, which are then sprouted and grown for a period of time so that a protein or polypeptide included in the transgenic sequences is produced in young plant tissues (*e.g.*, in sprouted seedlings). Typical technologies for the production of transgenic plant cells and/or seeds include *Agrobacterium tumefaciens* mediated gene transfer and microprojectile bombardment or electroporation.

[0113] Systems and reagents for generating a variety of sprouts and sprouted seedlings which are useful for production of HA polypeptide(s) as described herein have been described previously and are known in the art (see, for example, PCT Publication WO 04/43886). This document further provides sprouted seedlings, which

may be edible, as a biomass containing a HA polypeptide. In certain aspects, biomass is provided directly for consumption of antigen containing compositions. In some aspects, biomass is processed prior to consumption, for example, by homogenizing, crushing, drying, or extracting. In certain aspects, HA polypeptides are purified from biomass and formulated into a pharmaceutical composition.

[0114] Additionally provided are methods for producing HA polypeptide(s) in sprouted seedlings that can be consumed or harvested live (*e.g.*, sprouts, sprouted seedlings of the *Brassica* genus). In certain aspects, the methods can include growing a seed to an edible sprouted seedling in a contained, regulatable environment (*e.g.*, indoors and/or in a container). A seed can be a genetically engineered seed that contains an expression cassette encoding a HA polypeptide, which expression is driven by an exogenously inducible promoter. A variety of exogenously inducible promoters can be used that are inducible, for example, by light, heat, phytohormones, and/or nutrients.

[0115] In some embodiments, this document provides methods of producing HA polypeptide(s) in sprouted seedlings by first generating a seed stock for a sprouted seedling by transforming plants with an expression cassette that encodes HA polypeptide using an *Agrobacterium* transformation system, wherein expression of a HA polypeptide is driven by an inducible promoter. Transgenic seeds can be obtained from a transformed plant, grown in a contained, regulatable environment, and induced to express a HA polypeptide.

[0116] In some embodiments, methods are provided that include infecting sprouted seedlings with a viral expression cassette encoding a HA polypeptide, expression of which may be driven by any of a viral promoter or an inducible promoter. Sprouted seedlings can be grown for two to fourteen days in a contained, regulatable environment or at least until sufficient levels of HA polypeptide have been obtained for consumption or harvesting.

[0117] This document further provides systems for producing HA polypeptide(s) in sprouted seedlings that include a housing unit with climate control and a sprouted seedling containing an expression cassette that encodes one or more HA polypeptides, wherein expression is driven by a constitutive or inducible promoter. Systems can provide unique advantages over the outdoor environment or greenhouse, which cannot be controlled. Thus, this document enables a grower to precisely time the induction of expression of HA polypeptide. It can greatly reduce time and cost of producing HA polypeptide(s).

[0118] In certain aspects, transiently transfected sprouts contain viral vector sequences encoding an HA polypeptide as provided herein. Seedlings can be grown for a time period so as to allow for production of viral nucleic acid in sprouts, followed by a period of growth

wherein multiple copies of virus are produced, thereby resulting in production of HA polypeptide(s).

[0119] In certain aspects, genetically engineered seeds or embryos that contain a nucleic acid encoding HA polypeptide(s) are grown to sprouted seedling stage in a contained, regulatable environment. The contained, regulatable environment may be a housing unit or room in which seeds can be grown indoors. All environmental factors of a contained, regulatable environment may be controlled. Since sprouts do not require light to grow, and lighting can be expensive, genetically engineered seeds or embryos may be grown to sprouted seedling stage indoors in the absence of light.

[0120] Other environmental factors that can be regulated in a contained, regulatable environment include temperature, humidity, water, nutrients, gas (*e.g.*, O_2 or CO_2 content or air circulation), chemicals (small molecules such as sugars and sugar derivatives, or hormones such as phytohormones, including gibberellins and abscisic acid), and the like.

[0121] According to certain methods provided herein, expression of a nucleic acid encoding a HA polypeptide may be controlled by an exogenously inducible promoter. Exogenously inducible promoters are caused to increase or decrease expression of a nucleic acid in response to an external, rather than an internal stimulus. A number of environmental factors can act as inducers for expression of nucleic acids carried by expression cassettes of genetically engineered sprouts. A promoter may be a heat-inducible promoter, such as a heat-shock promoter. For example, using as heat-shock promoter, temperature of a contained environment may simply be raised to induce expression of a nucleic acid. Other promoters include light inducible promoters. Light-inducible promoters can be maintained as constitutive promoters if light in a contained regulatable environment is always on. Alternatively or additionally, expression of a nucleic acid can be turned on at a particular time during development by simply turning on the light. A promoter may be a chemically inducible promoter is used to induce expression of a nucleic acid. According to these embodiments, a chemical could simply be misted or sprayed onto seed, embryo, or seedling to induce expression of nucleic acid. Spraying and misting can be precisely controlled and directed onto target seed, embryo, or seedling to which it is intended. The contained environment is devoid of wind or air currents, which could disperse chemical away from intended target, so that the chemical stays on the target for which it was intended.

[0122] The time at which expression is induced can be selected to maximize expression of a HA polypeptide in sprouted seedling by the time of harvest. Inducing expression in an embryo at a particular stage of growth, for example, inducing expression in an embryo at a

particular number of days after germination, may result in maximum synthesis of a HA polypeptide at the time of harvest. For example, inducing expression from the promoter 4 days after germination may result in more protein synthesis than inducing expression from the promoter after 3 days or after 5 days. Those skilled in the art will appreciate that maximizing expression can be achieved by routine experimentation. In certain methods, sprouted seedlings are harvested at about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, or 12 days after germination.

[0123] In cases where the expression vector has a constitutive promoter instead of an inducible promoter, sprouted seedling may be harvested at a certain time after transformation of sprouted seedling. For example, if a sprouted seedling were virally transformed at an early stage of development, for example, at embryo stage, sprouted seedlings may be harvested at a time when expression is at its maximum post-transformation, *e.g.*, at about 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 14 days post-transformation. It could be that sprouts develop one, two, three or more months post-transformation, depending on germination of seed.

[0124] Generally, once expression of HA polypeptide(s) begins, seeds, embryos, or sprouted seedlings are allowed to grow until sufficient levels of HA polypeptide(s) are expressed. In certain aspects, sufficient levels are levels that would provide a therapeutic benefit to a patient if harvested biomass were eaten raw. Alternatively or additionally, sufficient levels are levels from which HA polypeptide can be concentrated or purified from biomass and formulated into a pharmaceutical composition that provides a therapeutic benefit to a patient upon administration. Typically, HA polypeptide is not a protein expressed in sprouted seedling in nature. At any rate, HA polypeptide is typically expressed at concentrations above that which would be present in the sprouted seedling in nature.

[0125] Once expression of HA polypeptide is induced, growth is allowed to continue until sprouted seedling stage, at which time sprouted seedlings are harvested. Sprouted seedlings can be harvested live. Harvesting live sprouted seedlings has several advantages including minimal effort and breakage. Sprouted seedlings may be grown hydroponically, making harvesting a simple matter of lifting a sprouted seedling from its hydroponic solution. No soil is required for growth of sprouted seedlings, but soil may be provided if deemed necessary or desirable by the skilled artisan. Because sprouts can be grown without soil, no cleansing of sprouted seedling material is required at the time of harvest. Being able to harvest the sprouted seedling directly from its hydroponic environment without washing or scrubbing minimizes breakage of harvested material. Breakage and wilting of plants induces

apoptosis. During apoptosis, certain proteolytic enzymes become active, which can degrade pharmaceutical protein expressed in the sprouted seedling, resulting in decreased therapeutic activity of the protein. Apoptosis-induced proteolysis can significantly decrease yield of protein from mature plants. Using methods provided herein, apoptosis may be avoided when no harvesting takes place until the moment proteins are extracted from the plant.

[0126] For example, live sprouts may be ground, crushed, or blended to produce a slurry of sprouted seedling biomass, in a buffer containing protease inhibitors. Buffer may be maintained at about 4°C. In some aspects, sprouted seedling biomass is air-dried, spray dried, frozen, or freeze-dried. As in mature plants, some of these methods, such as air-drying, may result in a loss of activity of pharmaceutical protein. However, because sprouted seedlings are very small and have a large surface area to volume ratio, this is much less likely to occur. Those skilled in the art will appreciate that many techniques for harvesting biomass that minimize proteolysis of expressed protein are available and could be applied to the subject matter described herein.

[0127] In some embodiments, sprouted seedlings are edible. In certain embodiments, sprouted seedlings expressing sufficient levels of HA polypeptides are consumed upon harvesting (*e.g.*, immediately after harvest, within minimal period following harvest) so that absolutely no processing occurs before sprouted seedlings are consumed. In this way, any harvest-induced proteolytic breakdown of HA polypeptide before administration of HA polypeptide to a patient in need of treatment is minimized. For example, sprouted seedlings that are ready to be consumed can be delivered directly to a patient. Alternatively or additionally, genetically engineered seeds or embryos are delivered to a patient in need of treatment and grown to sprouted seedling stage by a patient. In one aspect, a supply of genetically engineered sprouted seedlings is provided to a patient, or to a doctor who will be treating patients, so that a continual stock of sprouted seedlings expressing certain desirable HA polypeptide may be cultivated. This may be particularly valuable for populations in developing countries, where expensive pharmaceuticals are not affordable or deliverable. The ease with which sprouted seedlings can be grown can make them particularly desirable for such developing populations.

[0128] The regulatable nature of the contained environment can impart advantages over growing plants in the outdoor environment. In general, growing genetically engineered sprouted seedlings that express pharmaceutical proteins in plants provides a pharmaceutical product faster (because plants are harvested younger) and with less effort, risk, and regulatory

considerations than growing genetically engineered plants. A contained, regulatable environment reduces or eliminates risk of cross-pollinating plants in nature.

[0129] For example, a heat inducible promoter likely would not be used outdoors because outdoor temperature cannot be controlled. The promoter would be turned on any time the outdoor temperature rose above a certain level. Similarly, the promoter would be turned off every time the outdoor temperature dropped. Such temperature shifts could occur in a single day, for example, turning expression on in the daytime and off at night. A heat inducible promoter, such as those described herein, would not even be practical for use in a greenhouse, which is susceptible to climatic shifts to almost the same degree as outdoors. Growth of genetically engineered plants in a greenhouse is quite costly. In contrast, in the present system, every variable can be controlled so that the maximum amount of expression can be achieved with every harvest.

[0130] In certain embodiments, sprouted seedlings as provided herein can be grown in trays that can be watered, sprayed, or misted at any time during development of sprouted seedling. For example, a tray may be fitted with one or more watering, spraying, misting, and draining apparatus that can deliver and/or remove water, nutrients, and/or chemicals at specific time and at precise quantities during development of the sprouted seedling. For example, seeds require sufficient moisture to keep them damp. Excess moisture drains through holes in trays into drains in the floor of the room. Typically, drainage water is treated as appropriate for removal of harmful chemicals before discharge back into the environment.

[0131] Another advantage of trays is that they can be contained within a very small space. Since no light is required for sprouted seedlings to grow, trays containing seeds, embryos, or sprouted seedlings may be tightly stacked vertically on top of one another, providing a large quantity of biomass per unit floor space in a housing facility constructed specifically for these purposes. In addition, stacks of trays can be arranged in horizontal rows within the housing unit. Once seedlings have grown to a stage appropriate for harvest (about two to fourteen days) individual seedling trays are moved into a processing facility, either manually or by automatic means, such as a conveyor belt.

[0132] The system provided herein is unique in that it provides a sprouted seedling biomass, which is a source of a HA polypeptide(s). Whether consumed directly or processed into the form of a pharmaceutical composition, because sprouted seedlings are grown in a contained, regulatable environment, sprouted seedling biomass and/or pharmaceutical composition derived from biomass can be provided to a consumer at low cost. In addition, the fact that the conditions for growth of sprouted seedlings can be controlled makes the quality and purity of

product consistent. A contained, regulatable environment can obviate many safety regulations of the EPA that can prevent scientists from growing genetically engineered agricultural products outdoors.

Transformed Sprouts

[0133] A variety of methods can be used to transform plant cells and produce genetically engineered sprouted seedlings. Two available methods for transformation of plants that require that transgenic plant cell lines be generated *in vitro*, followed by regeneration of cell lines into whole plants include *Agrobacterium tumefaciens* mediated gene transfer and microprojectile bombardment or electroporation. In some embodiments, transient expression systems are utilized. Typical technologies for producing transient expression of proteins or polypeptides in plant tissues utilize plant viruses. Viral transformation provides more rapid and less costly methods of transforming embryos and sprouted seedlings that can be harvested without an experimental or generational lag prior to obtaining the desired product. For any of these techniques, the skilled artisan would appreciate how to adjust and optimize transformation protocols that have traditionally been used for plants, seeds, embryos, or spouted seedlings.

[0134] This document provides expression systems having advantages of viral expression systems (*e.g.*, rapid expression, high levels of production) and of *Agrobacterium* transformation (*e.g.*, controlled administration). In particular, as discussed in detail below, this document provides systems in which an agrobacterial construct (*i.e.*, a construct that replicates in *Agrobacterium* and therefore can be delivered to plant cells by delivery of *Agrobacterium*) includes a plant promoter that, after being introduced into a plant, directs expression of viral sequences (*e.g.*, including viral replication sequences) carrying a gene for a protein or polypeptide of interest. This system allows controlled, high level transient expression of proteins or polypeptides in plants.

[0135] A variety of different embodiments of expression systems, some of which produce transgenic plants and others of which provide for transient expression, are discussed in further detail individually below. For any of these techniques, the skilled artisan reading the present specification would appreciate how to adjust and optimize protocols for expression of proteins or polypeptides in young plant tissues (*e.g.*, sprouted seedlings).

Agrobacterium Transformation

[0136] *Agrobacterium* is a representative genus of the gram-negative family Rhizobiaceae. This species is responsible for plant tumors such as crown gall and hairy root disease. In dedifferentiated plant tissue, which is characteristic of tumors, amino acid derivatives known

as opines are produced by the *Agrobacterium* and catabolized by the plant. The bacterial genes responsible for expression of opines are a convenient source of control elements for chimeric expression cassettes. In some embodiments, an *Agrobacterium* transformation system may be used to generate young plants (*e.g.*, sprouted seedlings, including edible sprouted seedlings), which are merely harvested earlier than mature plants. *Agrobacterium* transformation methods can easily be applied to regenerate sprouted seedlings expressing HA polypeptides.

[0137] In general, transforming plants with *Agrobacterium* involves transformation of plant cells grown in tissue culture by co-cultivation with an *Agrobacterium tumefaciens* carrying a plant/bacterial vector. The vector contains a gene encoding a HA polypeptide. The *Agrobacterium* transfers vector to plant host cell and is then eliminated using antibiotic treatment. Transformed plant cells expressing HA polypeptide are selected, differentiated, and finally regenerated into complete plantlets (Hellens *et al.*, 2000, *Plant Mol. Biol.*, 42:819; Pilon-Smits *et al.*, 1999, *Plant Physiolog.*, 119:123; Barfield *et al.*, 1991, *Plant Cell Reports*, 10:308; and Riva *et al.*, 1998, *J. Biotech.*, 1(3)).

[0138] Agrobacterial expression vectors for use as described herein can include a gene (or expression cassette) encoding a HA polypeptide designed for operation in plants, with companion sequences upstream and downstream of the expression cassette. Companion sequences are generally of plasmid or viral origin and provide necessary characteristics to the vector to transfer DNA from bacteria to the desired plant host.

[0139] The basic bacterial/plant vector construct may desirably provide a broad host range prokaryote replication origin, a prokaryote selectable marker. Suitable prokaryotic selectable markers include resistance toward antibiotics such as ampicillin or tetracycline. Other DNA sequences encoding additional functions that are well known in the art may be present in the vector.

[0140] *Agrobacterium* T-DNA sequences are required for *Agrobacterium* mediated transfer of DNA to the plant chromosome. The tumor-inducing genes of T-DNA are typically removed during construction of an agrobacterial expression construct and are replaced with sequences encoding a HA polypeptide. T-DNA border sequences are retained because they initiate integration of the T-DNA region into the plant genome. If expression of HA polypeptide is not readily amenable to detection, the bacterial/plant vector construct may include a selectable marker gene suitable for determining if a plant cell has been transformed, *e.g.*, nptII kanamycin resistance gene. On the same or different bacterial/plant vector (Ti

plasmid) are Ti sequences. Ti sequences include virulence genes, which encode a set of proteins responsible for excision, transfer and integration of T-DNA into the plant genome (Schell, 1987, *Science*, 237:1176-86). Other sequences suitable for permitting integration of heterologous sequence into the plant genome may include transposon sequences, and the like, for homologous recombination.

[0141] On the same or different bacterial/plant vector (Ti plasmid) are Ti sequences. Ti sequences include the virulence genes, which encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome (Schell, 1987, *Science*, 237:1176-83). Other sequences suitable for permitting integration of the heterologous sequence into the plant genome may also include transposon sequences, and the like, for homologous recombination.

[0142] Certain constructs will include an expression cassette encoding an antigen protein. One, two, or more expression cassettes may be used in a given transformation. The recombinant expression cassette contains, in addition to a HA polypeptide encoding sequence, at least the following elements: a promoter region, plant 5' untranslated sequences, initiation codon (depending upon whether or not an expressed gene has its own), and transcription and translation termination sequences. In addition, transcription and translation terminators may be included in expression cassettes or chimeric genes. Signal secretion sequences that allow processing and translocation of a protein, as appropriate, may be included in the expression cassette.

[0143] A variety of promoters, signal sequences, and transcription and translation terminators are described, for example, in Lawton *et al.* (1987, *Plant Mol. Biol.*, 9:315-24) or in U.S. Patent 5,888,789. In addition, structural genes for antibiotic resistance are commonly utilized as a selection factor (Fraley *et al.*, 1983, *Proc. Natl. Acad. Sci., USA*, 80:4803-7). Unique restriction enzyme sites at the 5' and 3' ends of the cassette allow for easy insertion into a pre-existing vector.

[0144] Other binary vector systems for *Agrobacterium*-mediated transformation, carrying at least one T-DNA border sequence are described in PCT Publication WO 2000/020612. Further discussion of *Agrobacterium*-mediated transformation is found in Gelvin (2003, *Microbiol. Mol. Biol. Rev.*, 67:16-37) and Lorence and Verpoorte (2004, *Methods Mol. Biol.*, 267:329-50).

[0145] In certain embodiments, bacteria other than *Agrobacteria* are used to introduce a nucleic acid sequence into a plant. See, *e.g.*, Broothaerts *et al.* (2005, *Nature*, 433:629-33).

[0146] Seeds are prepared from plants that have been infected with *Agrobacteria* (or other bacteria) such that the desired heterologous gene encoding a protein or polypeptide of interest is introduced. Such seeds are harvested, dried, cleaned, and tested for viability and for the presence and expression of a desired gene product. Once this has been determined, seed stock is typically stored under appropriate conditions of temperature, humidity, sanitation, and security to be used when necessary. Whole plants may then be regenerated from cultured protoplasts, *e.g.*, as described in Evans *et al.* (*Handbook of Plant Cell Cultures*, Vol. 1, MacMillan Publishing Co., New York, NY, 1983); and in Vasil (ed., *Cell Culture and Somatic Cell Genetics of Plants*, Acad. Press, Orlando, FL, Vol. I, 1984, and Vol. III, 1986). In certain aspects, plants are regenerated only to sprouted seedling stage. In some aspects, whole plants are regenerated to produce seed stocks and sprouted seedlings are generated from seeds of the seed stock.

[0147] In certain embodiments, the plants are not regenerated into adult plants. For example, in some embodiments, plants are regenerated only to the sprouted seedling stage. In other embodiments, whole plants are regenerated to produce seed stocks and young plants (*e.g.*, sprouted seedlings) for use in accordance with the present disclosure, and are generated from the seeds of previously produced seed stock.

[0148] All plants from which protoplasts can be isolated and cultured to give whole, regenerated plants can be transformed by *Agrobacteria* so that whole plants are recovered that contain a transferred gene. It is known that practically all plants can be regenerated from cultured cells or tissues, including, but not limited to, all major species of plants that produce edible sprouts. Some suitable plants include alfalfa, mung bean, radish, wheat, mustard, spinach, carrot, beet, onion, garlic, celery, rhubarb, leafy plants such as cabbage and lettuce, watercress or cress, herbs such as parsley, mint, and clover, cauliflower, broccoli, soybean, lentils, and edible flowers such as sunflower.

[0149] Means for regeneration of plants from transformed cells vary from one species of plants to the next. However, those skilled in the art will appreciate that generally a suspension of transformed protoplants containing copies of a heterologous gene is first provided. Callus tissue is formed and shoots may be induced from callus and subsequently rooted. Alternatively or additionally, embryo formation can be induced from a protoplast suspension. These embryos germinate as natural embryos to form plants. Steeping seed in

water or spraying seed with water to increase the moisture content of the seed to between 35% – 45% initiates germination. For germination to proceed, seeds are typically maintained in air saturated with water under controlled temperature and airflow conditions. The culture media will generally contain various amino acids and hormones, such as auxin and cytokinins. It is advantageous to add glutamic acid and proline to the medium, especially for such species as alfalfa. Shoots and roots normally develop simultaneously. Efficient regeneration will depend on the medium, the genotype, and the history of the culture. If these three variables are controlled, then regeneration is fully reproducible and repeatable.

[0150] Mature plants, grown from the transformed plant cells, are selfed and non-segregating, homozygous transgenic plants are identified. The inbred plant produces seeds containing antigen-encoding sequences. Such seeds can be germinated and grown to sprouted seedling stage to produce HA polypeptide(s) as provided herein.

[0151] In related embodiments, transgenic seeds (*e.g.*, carrying the transferred gene encoding a HA polypeptide, typically integrated into the genome) may be formed into seed products and sold with instructions on how to grow young plants to the appropriate stage (*e.g.*, sprouted seedling stage) for harvesting and/or administration or harvesting into a formulation as described herein. In some related embodiments, hybrids or novel varieties embodying desired traits may be developed from inbred plants.

Direct Integration

[0152] Direct integration of DNA fragments into the genome of plant cells by microprojectile bombardment or electroporation may also be used to introduce expression constructs encoding HA polypeptides into plant tissues (see, *e.g.*, Kikkert, *et al.*, 1999, *Plant: J. Tiss. Cult. Assoc.*, 35:43; and Bates, 1994, *Mol. Biotech.*, 2:135). More particularly, vectors that express HA polypeptide(s) can be introduced into plant cells by a variety of techniques. As described above, vectors may include selectable markers for use in plant cells. Vectors may include sequences that allow their selection and propagation in a secondary host, such as sequences containing an origin of replication and selectable marker. Typically, secondary hosts include bacteria and yeast. In some embodiments, a secondary host is bacteria (*e.g.*, *Escherichia coli*, the origin of replication is a colE1-type origin of replication) and a selectable marker is a gene encoding ampicillin resistance. Such sequences are well known in the art and are commercially available (*e.g.*, Clontech, Palo Alto, CA or Stratagene, La Jolla, CA).

[0153] Vectors as provided herein may be modified to intermediate plant transformation plasmids that contain a region of homology to an *Agrobacterium tumefaciens* vector, a T-

DNA border region from *Agrobacterium tumefaciens*, and chimeric genes or expression cassettes described above. Further vectors may include a disarmed plant tumor inducing plasmid of *Agrobacterium tumefaciens*.

[0154] According to some embodiments, direct transformation of vectors can involve microinjecting vectors directly into plant cells by use of micropipettes to mechanically transfer recombinant DNA (see, *e.g.*, Crossway, 1985, *Mol. Gen. Genet.*, 202:179). Genetic material may be transferred into a plant cell using polyethylene glycols (see, *e.g.*, Krens *et al.*, 1982, *Nature* 296:72). Another method of introducing nucleic acids into plants via high velocity ballistic penetration by small particles with a nucleic acid either within the matrix of small beads or particles, or on the surface (see, *e.g.*, Klein *et al.*, 1987, *Nature* 327:70; and Knudsen *et al.*, *Planta*, 185:330). Yet another method of introduction is fusion of protoplasts with other entities, either minicells, cells, lysosomes, or other fusible lipid-surfaced bodies (see, *e.g.*, Fraley *et al.*, 1982, *Proc. Natl. Acad. Sci., USA*, 79:1859). Vectors in accordance with this document may be introduced into plant cells by electroporation (see, *e.g.*, Fromm *et al.* 1985, *Proc. Natl. Acad. Sci., USA*, 82:5824). According to this technique, plant protoplasts are electroporated in the presence of plasmids containing a gene construct. Electrical impulses of high field strength reversibly permeabilize biomembranes allowing introduction of plasmids. Electroporated plant protoplasts reform the cell wall divide and form plant callus, which can be regenerated to form sprouted seedlings. Those skilled in the art will appreciate how to utilize these methods to transform plants cells that can be used to generate edible sprouted seedlings.

Viral Transformation

[0155] Similar to conventional expression systems, plant viral vectors can be used to produce full-length proteins, including full length antigen. Plant virus vectors may be used to infect and produce antigen(s) in seeds, embryos, or sprouted seedlings, for example. In this regard infection includes any method of introducing a viral genome, or portion thereof, into a cell, including, but not limited to, the natural infectious process of a virus, abrasion, and inoculation. The term includes introducing a genomic RNA transcript, or a cDNA copy thereof, into a cell. The viral genome need not be a complete genome but will typically contain sufficient sequences to allow replication. The genome may encode a viral replicase and may contain any cis-acting nucleic acid elements necessary for replication. Expression of high levels of foreign genes encoding short peptides as well as large complex proteins

(*e.g.*, by tobamoviral vectors) is described (see, *e.g.*, McCormick *et al.*, 1999, *Proc. Natl. Acad. Sci., USA*, 96:703; Kumagai *et al.* 2000, *Gene*, 245:169; and Verch *et al.*, 1998, *J. Immunol. Methods*, 220:69). Thus, plant viral vectors have a demonstrated ability to express short peptides as well as large complex proteins.

[0156] In certain embodiments, young plants (*e.g.*, sprouts), which express HA polypeptide, are generated utilizing a host/virus system. Young plants produced by viral infection provide a source of transgenic protein that has already been demonstrated to be safe. For example, sprouts are free of contamination with animal pathogens. Unlike, for example, tobacco, proteins from an edible sprout could at least in theory be used in oral applications without purification, thus significantly reducing costs.

[0157] In addition, a virus/young plant (*e.g.*, sprout) system offers a much simpler, less expensive route for scale-up and manufacturing, since the relevant genes (encoding the protein or polypeptide of interest) are introduced into the virus, which can be grown up to a commercial scale within a few days. In contrast, transgenic plants can require up to 5-7 years before sufficient seeds or plant material is available for large-scale trials or commercialization.

[0158] As described herein, plant RNA viruses can have certain advantages, which make them attractive as vectors for foreign protein expression. The molecular biology and pathology of a number of plant RNA viruses are well characterized and there is considerable knowledge of virus biology, genetics, and regulatory sequences. Most plant RNA viruses have small genomes and infectious cDNA clones are available to facilitate genetic manipulation. Once infectious virus material enters a susceptible host cell, it replicates to high levels and spreads rapidly throughout the entire sprouted seedling (one to ten days post inoculation, *e.g.*, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, or more than 10 days post-inoculation). Virus particles are easily and economically recovered from infected sprouted seedling tissue. Viruses have a wide host range, enabling use of a single construct for infection of several susceptible species. These characteristics are readily transferable to sprouts.

[0159] Foreign sequences can be expressed from plant RNA viruses, typically by replacing one of the viral genes with desired sequence, by inserting foreign sequences into the virus genome at an appropriate position, or by fusing foreign peptides to structural proteins of a virus. Moreover, any of these approaches can be combined to express foreign sequences by trans-complementation of vital functions of a virus. A number of different strategies exist as

tools to express foreign sequences in virus-infected plants using tobacco mosaic virus (TMV), alfalfa mosaic virus (AlMV), and chimeras thereof.

[0160] The genome of AlMV is a representative of the *Bromoviridae* family of viruses and consists of three genomic RNAs (RNAs1-3) and subgenomic RNA (RNA4). Genomic RNAs1 and 2 encode virus replicase proteins P1 and 2, respectively. Genomic RNA3 encodes cell-to-cell movement protein P3 and coat protein (CP). CP is translated from subgenomic RNA4, which is synthesized from genomic RNA3, and is required to start infection. Studies have demonstrated the involvement of CP in multiple functions, including genome activation, replication, RNA stability, symptom formation, and RNA encapsidation (see *e.g.*, Bol *et al.*, 1971, *Virology*, 46:73; Van Der Vossen *et al.*, 1994, *Virology* 202:891; Yusibov *et al.*, *Virology*, 208:405; Yusibov *et al.*, 1998, *Virology*, 242:1; Bol *et al.*, (Review, 100 refs.), 1999, *J. Gen. Virol.*, 80:1089; De Graaff, 1995, *Virology*, 208:583; Jaspars *et al.*, 1974, *Adv. Virus Res.*, 19:37; Loesch-Fries, 1985, *Virology*, 146:177; Neeleman *et al.*, 1991, *Virology*, 181:687; Neeleman *et al.*, 1993, *Virology*, 196: 883; Van Der Kuyl *et al.*, 1991, *Virology*, 183:731; and Van Der Kuyl *et al.*, 1991, *Virology*, 185:496).

[0161] Encapsidation of viral particles is typically required for long distance movement of virus from inoculated to un-inoculated parts of seed, embryo, or sprouted seedling and for systemic infection. Inoculation can occur at any stage of plant development. In embryos and sprouts, spread of inoculated virus should be very rapid. Virions of AlMV are encapsidated by a unique CP (24 kD), forming more than one type of particle. The size (30- to 60-nm in length and 18 nm in diameter) and shape (spherical, ellipsoidal, or bacilliform) of the particle depends on the size of the encapsidated RNA. Upon assembly, the N-terminus of AlMV CP is thought to be located on the surface of the virus particles and does not appear to interfere with virus assembly (Bol *et al.*, 1971, *Virology*, 6:73).

Additionally, ALMV CP with an additional 38-amino acid peptide at its N-terminus forms particles *in vitro* and retains biological activity (Yusibov *et al.*, 1995, *J. Gen. Virol.*, 77:567).

[0162] AlMV has a wide host range, which includes a number of agriculturally valuable crop plants, including plant seeds, embryos, and sprouts. Together, these characteristics make ALMV CP an excellent candidate as a carrier molecule and AlMV an attractive candidate vector for expression of foreign sequences in a plant at the sprout stage of development. Moreover, upon expression from a heterologous vector such as TMV, AlMV CP encapsidates TMV genome without interfering with virus infectivity (Yusibov *et al.*, 1997, *Proc. Natl.*

Acad. Sci., USA, 94:5784). This allows use of TMV as a carrier virus for AlMV CP fused to foreign sequences.

[0163] TMV, the prototype of tobamoviruses, has a genome consisting of a single plus-sense RNA encapsidated with a 17.0 kD CP, which results in rod-shaped particles (300 nm in length). CP is the only structural protein of TMV and is required for encapsidation and long distance movement of virus in an infected host (Saito *et al.*, 1990, *Virology* 176:329). 183 and 126 kD proteins are translated from genomic RNA and are required for virus replication (Ishikawa *et al.*, 1986, *Nucleic Acids Res.*, 14:8291). 30 kD protein is the cell-to-cell movement protein of virus (Meshi *et al.*, 1987, *EMBO J.*, 6:2557). Movement and coat proteins are translated from subgenomic mRNAs (Hunter *et al.*, 1976, *Nature*, 260:759; Bruening *et al.*, 1976, *Virology*, 71:498; and Beachy *et al.*, 1976, *Virology*, 73:498).

[0164] Other methods that may be utilized to introduce a gene encoding a HA polypeptide into plant cells include transforming the flower of a plant. Transformation of *Arabidopsis thaliana* can be achieved by dipping plant flowers into a solution of *Agrobacterium tumefaciens* (Curtis *et al.*, 2001, *Transgenic Res.*, 10:363; and Qing *et al.*, 2000, *Molecular Breeding: New Strategies in Plant Improvement* 1:67). Transformed plants are formed in the population of seeds generated by "dipped" plants. At a specific point during flower development, a pore exists in the ovary wall through which *Agrobacterium tumefaciens* gains access to the interior of the ovary. Once inside the ovary, the *Agrobacterium tumefaciens* proliferates and transforms individual ovules (Desfeux *et al.*, 2000, *Plant Physiology*, 123:895). Transformed ovules follow the typical pathway of seed formation within the ovary.

Agrobacterium-Mediated Transient Expression

[0165] As indicated herein, systems for rapid (*e.g.*, transient) expression of proteins or polypeptides in plants can be desirable. Among other things, this document provides a powerful system for achieving such rapid expression in plants (particularly in young plants, *e.g.*, sprouted seedlings) that utilizes an agrobacterial construct to deliver a viral expression system encoding a HA polypeptide.

[0166] Specifically, as described herein, a "launch vector" is prepared that contains agrobacterial sequences including replication sequences and also contains plant viral sequences (including self-replication sequences) that carry a gene encoding the protein or polypeptide of interest. A launch vector is introduced into plant tissue, preferably by

agroinfiltration, which allows substantially systemic delivery. For transient transformation, non-integrated T-DNA copies of the launch vector remain transiently present in the nucleolus and are transcribed leading to the expression of the carrying genes (Kapila *et al.*, 1997, *Plant Science*, 122:101-108). *Agrobacterium*-mediated transient expression, differently from viral vectors, cannot lead to the systemic spreading of the expression of the gene of interest. One advantage of this system is the possibility to clone genes larger than 2 kb to generate constructs that would be impossible to obtain with viral vectors (Voinnet *et al.*, 2003, *Plant J.*, 33:949-56).

Furthermore, using such technique, it is possible to transform the plant with more than one transgene, such that multimeric proteins (*e.g.*, antibodies subunits of complexed proteins) can be expressed and assembled. Furthermore, the possibility of co-expression of multiple transgenes by means of co-infiltration with different *Agrobacterium* can be taken advantage of, either by separate infiltration or using mixed cultures.

[0167] In certain embodiments, a launch vector includes sequences that allow for selection (or at least detection) in *Agrobacteria* and also for selection/detection in infiltrated tissues. Furthermore, a launch vector typically includes sequences that are transcribed in the plant to yield viral RNA production, followed by generation of viral proteins. Furthermore, production of viral proteins and viral RNA yields rapid production of multiple copies of RNA encoding the pharmaceutically active protein of interest. Such production results in rapid protein production of the target of interest in a relatively short period of time. Thus, a highly efficient system for protein production can be generated.

[0168] The agroinfiltration technique utilizing viral expression vectors can be used to produce limited quantity of protein of interest in order to verify the expression levels before deciding if it is worth generating transgenic plants. Alternatively or additionally, the agroinfiltration technique utilizing viral expression vectors is useful for rapid generation of plants capable of producing huge amounts of protein as a primary production platform. Thus, this transient expression system can be used on industrial scale.

[0169] Further provided are any of a variety of different *Agrobacterial* plasmids, binary plasmids, or derivatives thereof such as pBIV, pBI1221, and pGreen, which can be used in these and other aspects of the present disclosure. Numerous suitable vectors are known in the art and can be directed and/or modified according to methods known in the art, or those described herein so as to utilize in the methods described provided herein.

[0170] An exemplary launch vector, pBID4, contains the 35S promoter of cauliflower mosaic virus (a DNA plant virus) that drives initial transcription of the recombinant viral genome

following introduction into plants, and the *nos* terminator, the transcriptional terminator of *Agrobacterium* nopaline synthase. The vector further contains sequences of the tobacco mosaic virus genome including genes for virus replication (126/183K) and cell-t-cell movement (MP). The vector further contains a gene encoding a polypeptide of interest, inserted into a unique cloning site within the tobacco mosaic virus genome sequences and under the transcriptional control of the coat protein subgenomic mRNA promoter. Because this "target gene" (*i.e.*, gene encoding a protein or polypeptide of interest) replaces coding sequences for the TMV coat protein, the resultant viral vector is naked self-replicating RNA that is less subject to recombination than CP-containing vectors, and that cannot effectively spread and survive in the environment. Left and right border sequences (LB and RB) delimit the region of the launch vector that is transferred into plant cells following infiltration of plants with recombinant *Agrobacterium* carrying the vector. Upon introduction of agrobacteria carrying this vector into plant tissue (typically by agroinfiltration but alternatively by injection or other means), multiple single-stranded DNA (ssDNA) copies of sequence between LB and RB are generated and released in a matter of minutes. These introduced sequences are then amplified by viral replication. Translation of the target gene results in accumulation of large amounts of target protein or polypeptide in a short period of time.

[0171] In some embodiments, *Agrobacterium*-mediated transient expression produces up to about 5 g or more of target protein per kg of plant tissue. For example, in some embodiments, up to about 4 g, about 3 g, about 2 g, about 1 g, or about 0.5 g of target protein is produced per kg of plant tissue. In some embodiments, at least about 20 mg to about 500 mg, or about 50 mg to about 500 mg of target protein, or about 50 mg to about 200 mg, or about 50 mg, about 60 mg, about 70 mg, about 80 mg, about 90 mg, about 100 mg, about 110 mg, about 120 mg, about 130 mg, about 140 mg, about 150 mg, about 160 mg, about 170 mg, about 180 mg, about 190 mg, about 200 mg, about 250 mg, about 300 mg, about 350 mg, about 400 mg, about 450 mg, about 500 mg, about 550 mg, about 600 mg, about 650 mg, about 700 mg, about 750 mg, about 800 mg, about 850 mg, about 900 mg, about 950 mg, about 1000 mg, about 1500 mg, about 1750 mg, about 2000 mg, about 2500 mg, about 3000 mg or more of protein per kg of plant tissue is produced.

[0172] In some embodiments, these expression levels are achieved within about 6, about 5, about 4, about 3, or about 2 weeks from infiltration. In some embodiments, these expression levels are achieved within about 10, about 9, about 8, about 7, about 6, about 5, about 4, about 3, about 2 days, or even about 1 day, from introduction of the expression construct.

Thus, the time from introduction (*e.g.*, infiltration) to harvest is typically less than about 2 weeks, about 10 days, about 1 week or less. This allows production of protein within about 8 weeks or less from the selection of amino acid sequence (even including time for "preliminary" expression studies). Also, each batch of protein can typically be produced within about 8 weeks, about 6 weeks, about 5 weeks, or less. Those of ordinary skill in the art will appreciate that these numbers may vary somewhat depending on the type of plant used. Most sprouts, including peas, will fall within the numbers given. *Nicotiana benthamiana*, however, may be grown longer, particularly prior to infiltration, as they are slower growing (from a much smaller seed). Other expected adjustments will be clear to those of ordinary skill in the art based on biology of the particular plants utilized.

[0173] A launch vector system has been used to produce a variety of target proteins and polypeptides in a variety of different young plants. In some embodiments, certain pea varieties including for example, marrowfat pea, bill jump pea, yellow trapper pea, speckled pea, and green pea are particularly useful in the practice of methods as disclosed herein, for example.

[0174] Various *Nicotiana* plants can be particularly useful in the practice of this disclosure, including in particular *Nicotiana benthamiana.* It will be understood by those of ordinary skill in the art that *Nicotiana* plants are generally not considered to be "sprouts." Nonetheless, young *Nicotiana* plants (particularly young *Nicotiana benthamiana* plants) can be useful in the practices provided herein. In general, in some embodiments, *Nicotiana benthamiana* plants are grown for a time sufficient to allow development of an appropriate amount of biomass prior to infiltration (*i.e.*, to delivery of agrobacteria containing the launch vector). Typically, the plants are grown for a period of more than about 3 weeks, more typically more than about 4 weeks, or between about 5 to about 6 weeks to accumulate biomass prior to infiltration.

[0175] It has further surprisingly been found that, although both TMV and AlMV sequences can prove effective in such launch vector constructs, in some embodiments, AlMV sequences are particularly efficient at ensuring high level production of delivered protein or polypeptides.

[0176] Thus, in some embodiments, proteins or polypeptides of interest can be produced in young pea plants or young *Nicotania* plants (*e.g.*, *Nicotiana benthamiana*) from a launch vector that directs production of AlMV sequences carrying the gene of interest.

Expression Constructs

[0177] Many features of expression constructs useful as described herein will be specific to the particular expression system used, as discussed above. However, certain aspects that may be applicable across different expression systems are discussed in further detail here.

[0178] To give but one example, in some embodiments, it will be desirable that expression of the protein or polypeptide (or nucleic acid) of interest be inducible. In many such embodiments, production of an RNA encoding the protein or polypeptide of interest (and/or production of an antisense RNA) is under the control of an inducible (*e.g.* exogenously inducible) promoter. Exogenously inducible promoters are caused to increase or decrease expression of a transcript in response to an external, rather than an internal stimulus. A number of environmental factors can act as such an external stimulus. In certain embodiments, transcription is controlled by a heat-inducible promoter, such as a heat-shock promoter.

[0179] Externally inducible promoters may be particularly useful in the context of controlled, regulatable growth settings. For example, using a heat-shock promoter the temperature of a contained environment may simply be raised to induce expression of the relevant transcript. In will be appreciated, of course, that a heat inducible promoter could never be used in the outdoors because the outdoor temperature cannot be controlled. The promoter would be turned on any time the outdoor temperature rose above a certain level. Similarly, the promoter would be turned off every time the outdoor temperature dropped. Such temperature shifts could occur in a single day, for example, turning expression on in the daytime and off at night. A heat inducible promoter, such as those described herein, would likely not even be practical for use in a greenhouse, which is susceptible to climatic shifts to almost the same degree as the outdoors. Growth of genetically engineered plants in a greenhouse is quite costly. In contrast, in the present system, every variable can be controlled so that the maximum amount of expression can be achieved with every harvest.

[0180] Other externally-inducible promoters than can be utilized include light inducible promoters. Light-inducible promoters can be maintained as constitutive promoters if the light in the contained regulatable environment is always on. Alternatively, expression of the relevant transcript can be turned on at a particular time during development by simply turning on the light.

[0181] In yet other embodiments, a chemically inducible promoter is used to induce expression of the relevant transcript. According to these embodiments, the chemical could simply be misted or sprayed onto a seed, embryo, or young plant (*e.g.*, seedling) to induce expression of the relevant transcript. Spraying and misting can be precisely controlled and

directed onto a particular seed, embryo, or young plant (*e.g.*, seedling) as desired. A contained environment is devoid of wind or air currents, which could disperse the chemical away from the intended recipient, so that the chemical stays on the recipient for which it was intended.

Production and Isolation of Antigen

[0182] In general, standard methods known in the art may be used for culturing or growing plants, plant cells, and/or plant tissues (*e.g.*, clonal plants, clonal plant cells, clonal roots, clonal root lines, sprouts, sprouted seedlings, and plants) for production of antigen(s). A wide variety of culture media and bioreactors have been employed to culture hairy root cells, root cell lines, and plant cells (see, for example, Giri *et al.*, 2000, *Biotechnol. Adv.*, 18:1; Rao *et al.*, 2002, *Biotechnol. Adv.*, 20:101; and references in both of the foregoing).

Clonal plants may be grown in any suitable manner.

[0183] In a certain embodiments, HA polypeptides as provided herein can be produced by any known method. In some embodiments, a HA polypeptide is expressed in a plant or portion thereof. Proteins are isolated and purified in accordance with conventional conditions and techniques known in the art. These include methods such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, and the like. Thus, this document provides methods that include purification and affordable scaling up of production of HA polypeptide(s) using any of a variety of plant expression systems known in the art and provided herein, including viral plant expression systems described herein.

[0184] In some embodiments, it can be desirable to isolate HA polypeptide(s) for vaccine products. Where a protein is produced from plant tissue(s) or a portion thereof, *e.g.*, roots, root cells, plants, plant cells, that express them, methods described in further detail herein, or any applicable methods known in the art may be used for any of partial or complete isolation from plant material. Where it is desirable to isolate the expression product from some or all of plant cells or tissues that express it, any available purification techniques may be employed. Those of ordinary skill in the art are familiar with a wide range of fractionation and separation procedures (see, for example, Scopes *et al.*, *Protein Purification: Principles and Practice*, 3rd Ed., Janson *et al.*, 1993; *Protein Purification: Principles, High Resolution Methods, and Applications*, Wiley-VCH, 1998; Springer-Verlag, NY, 1993; and Roe, *Protein Purification Techniques*, Oxford University Press, 2001).

Often, it will be desirable to render the product more than about 50%, about 60%, about 70%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%,

about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% pure. See, *e.g.*, U.S. Patents 6,740,740 and 6,841,659

for discussion of certain methods useful for purifying substances from plant tissues or fluids.

[0185] Those skilled in the art will appreciate that a method of obtaining desired HA polypeptide(s) product(s) is by extraction. Plant material (*e.g.*, roots and/or leaves) may be extracted to remove desired products from residual biomass, thereby increasing the concentration and purity of product. Plants may be extracted in a buffered solution. For example, plant material may be transferred into an amount of ice-cold water at a ratio of one to one by weight that has been buffered with, *e.g.*, phosphate buffer. Protease inhibitors can be added as required. The plant material can be disrupted by vigorous blending or grinding while suspended in buffer solution and extracted biomass removed by filtration or centrifugation. The product carried in solution can be further purified by additional steps or converted to a dry powder by freeze-drying or precipitation. Extraction can be carried out by pressing. Plants or roots can be extracted by pressing in a press or by being crushed as they are passed through closely spaced rollers. Fluids expressed from crushed plants or roots are collected and processed according to methods well known in the art. Extraction by pressing allows release of products in a more concentrated form. However, overall yield of product may be lower than if product were extracted in solution.

[0186] In some embodiments, produced proteins or polypeptides are not isolated from plant tissue but rather are provided in the context of live plants (*e.g.*, sprouted seedlings). In some embodiments, where the plant is edible, plant tissue containing expressed protein or polypeptide is provided directly for consumption. Thus, this document provides edible young plant biomass (*e.g.*, edible sprouted seedlings) containing expressed protein or polypeptide.

[0187] Where edible plants (*e.g.*, sprouted seedlings) express sufficient levels of pharmaceutical proteins or polypeptides and are consumed live, in some embodiments absolutely no harvesting occurs before the sprouted seedlings are consumed. In this way, it is guaranteed that there is no harvest-induced proteolytic breakdown of the pharmaceutical protein before administration of the pharmaceutical protein to a patient in need of treatment. For example, young plants (*e.g.*, sprouted seedlings) that are ready to be consumed can be delivered directly to a patient. Alternatively, genetically engineered seeds or embryos are delivered to a patient in need of treatment and grown to the sprouted seedling stage by the patient. In some embodiments, a supply of genetically engineered sprouted seedlings is provided to a patient, or to a doctor who will be treating patients, so that a continual stock of sprouted seedlings expressing certain desirable pharmaceutical proteins may be cultivated.

This may be particularly valuable for populations in developing countries, where expensive pharmaceuticals are not affordable or deliverable. The ease with which the sprouted seedlings can be grown can make them particularly desirable for such developing populations.

[0188] In some embodiments, plant biomass is processed prior to consumption or formulation, for example, by homogenizing, crushing, drying, or extracting. In some embodiments, the expressed protein or polypeptide is isolated or purified from the biomass and formulated into a pharmaceutical composition.

[0189] For example, live plants (*e.g.*, sprouts) may be ground, crushed, or blended to produce a slurry of biomass, in a buffer containing protease inhibitors. Preferably the buffer is at about 4°C. In certain embodiments, the biomass is air-dried, spray dried, frozen, or freeze-dried. As in mature plants, some of these methods, such as air-drying, may result in a loss of activity of the pharmaceutical protein or polypeptide. However, because plants (*e.g.*, sprouted seedlings) may be very small and typically have a large surface area to volume ratio, this is much less likely to occur. Those skilled in the art will appreciate that many techniques for harvesting the biomass that minimize proteolysis of the pharmaceutical protein or polypeptide are available and could be applied to the methods provided herein.

Antibodies

[0190] This document also provides pharmaceutical antigen and antibody proteins for therapeutic use, such as influenza antigen(s) (*e.g.*, influenza protein(s) or an immunogenic portion(s) thereof, or fusion proteins comprising influenza antibody protein(s) or an antigen binding portion(s) thereof) active as antibody for therapeutic and/or prophylactic treatment of influenza infection. Further, this document provides veterinary uses, as such influenza antigen is active in veterinary applications. In certain embodiments, influenza antigen(s) and/or antibodies may be produced by plant(s) or portion(s) thereof (*e.g.*, root, cell, sprout, cell line, or plant) as described herein. In certain embodiments, provided influenza antigens and/or antibodies are expressed in plants, plant cells, and/or plant tissues (*e.g.*, sprouts, sprouted seedlings, roots, root culture, clonal cells, clonal cell lines, or clonal plants), and can be used directly from plant or partially purified or purified in preparation for pharmaceutical administration to a subject.

Monoclonal Antibodies

[0191] Various methods for generating monoclonal antibodies (MAbs) are now very well known in the art. The most standard monoclonal antibody generation techniques generally

begin along the same lines as those for preparing polyclonal antibodies (*Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988).

A polyclonal antibody response is initiated by immunizing an animal with an immunogenic anionic phospholipid and/or aminophospholipid composition and, when a desired titer level is obtained, the immunized animal can be used to generate MAbs. Typically, the particular screening and selection techniques disclosed herein are used to select antibodies with the sought after properties.

[0192] MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265. Typically, the technique involves immunizing a suitable animal with a selected immunogen composition to stimulate antibody producing cells. Rodents such as mice and rats are exemplary animals, however, the use of rabbit, sheep and frog cells is possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are sometimes preferred, with the BALB/c mouse often being most preferred as this is most routinely used and generally gives a higher percentage of stable fusions.

[0193] Following immunization, somatic cells with the potential for producing the desired antibodies, specifically B lymphocytes (B cells), are selected for use in the MAb generation and fusion with cells of an immortal myeloma cell, generally one of the same species as the animal that was immunized. Myeloma cell lines suited for use in hybridoma-producing fusion procedures typically are non-antibody-producing, have high fusion efficiency, and enzyme deficiencies that render then incapable of growing in certain selective media which support the growth of only the desired fused cells (hybridomas). Any one of a number of myeloma cells may be used, as are known to those of skill in the art (Goding, pp. 65-66, 1986; Campbell, pp. 75-83, 1984). For example, where the immunized animal is a mouse, one may use P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO, NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F, 4B210 or one of the above listed mouse cell lines; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6, are all useful in connection with human cell fusions.

[0194] This culturing provides a population of hybridomas from which specific hybridomas are selected, followed by serial dilution and cloning into individual antibody producing lines, which can be propagated indefinitely for production of antibody.

[0195] MAbs produced are generally be further purified, *e.g.*, using filtration, centrifugation and various chromatographic methods, such as HPLC or affinity chromatography, all of

which purification techniques are well known to those of skill in the art. These purification techniques each involve fractionation to separate the desired antibody from other components of a mixture. Analytical methods particularly suited to the preparation of antibodies include, for example, protein A-Sepharose™ and/or protein G-Sepharose chromatography.

Antibody Fragments and Derivatives

[0196] Irrespective of the source of the original antibody against a hemagglutinin, either the intact antibody, antibody multimers, or any one of a variety of functional, antigen-binding regions of the antibody may be used. Exemplary functional regions include scFv, Fv, Fab', Fab and F(ab').sub.2 fragments of antibodies. Techniques for preparing such constructs are well known to those in the art and are further exemplified herein.

[0197] The choice of antibody construct may be influenced by various factors. For example, prolonged half-life can result from the active readsorption of intact antibodies within the kidney, a property of the Fc piece of immunoglobulin. IgG based antibodies, therefore, are expected to exhibit slower blood clearance than their Fab' counterparts. However, Fab' fragment-based compositions will generally exhibit better tissue penetrating capability.

[0198] Antibody fragments can be obtained by proteolysis of the whole immunoglobulin by the non-specific thiolprotease, papain. Papain digestion yields two identical antigen-binding fragments, termed "Fab fragments," each with a single antigen-binding site, and a residual "Fc fragment." The various fractions are separated by protein A-Sepharose or ion exchange chromatography.

[0199] The usual procedure for preparation of F(ab').sub.2 fragments from IgG of rabbit and human origin is limited proteolysis by the enzyme pepsin. Pepsin treatment of intact antibodies yields an F(ab').sub.2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

[0200] A Fab fragment contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteine(s) from the antibody hinge region. F(ab').sub.2 antibody fragments were originally produced as pairs of Fab' fragments that have hinge cysteines between them. Other chemical couplings of antibody fragments are known.

[0201] An "Fv" fragment is the minimum antibody fragment that contains a complete antigen-recognition and binding site. This region consists of a dimer of one heavy chain and one light chain variable domain in tight, con-covalent association. It is in this configuration that three hypervariable regions of each variable domain interact to define an antigen-binding

site on the surface of the V_H-V_L dimer. Collectively, six hypervariable regions confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three hypervariable regions specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

[0202] "Single-chain Fv" or "scFv" antibody fragments (now known as "single chains") comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between V_H and V_L domains that enables sFv to form the desired structure for antigen binding.

[203] The following patents are mentioned for the purposes of even further supplementing the present teachings regarding the preparation and use of functional, antigen-binding regions of antibodies, including scFv, Fv, Fab', Fab and F(ab').sub.2 fragments of antibodies: U.S. Patents 5,855,866; 5,877,289; 5,965,132; 6,093,399; 6,261,535; and 6,004,555. WO 98/45331 is also mentioned for the purposes including even further describing and teaching the preparation of variable, hypervariable and complementarity determining (CDR) regions of antibodies.

[0204] "Diabodies" are small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (V_H) connected to a light chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described in EP 404,097 and WO 93/11161.

"Linear antibodies," which can be bispecific or monospecific, comprise a pair of tandem Fd segments (V.sub.H-C.sub.H1-V.sub.H-C.sub.H1) that form a pair of antigen binding regions, as described (see, for example, Zapata *et al.* ,1995).

[0205] In using a Fab' or antigen binding fragment of an antibody, with the attendant benefits on tissue penetration, one may derive additional advantages from modifying the fragment to increase its half-life. A variety of techniques may be employed, such as manipulation or modification of the antibody molecule itself, and conjugation to inert carriers. Any conjugation for the sole purpose of increasing half-life, rather than to deliver an agent to a target, should be approached carefully in that Fab' and other fragments are chosen to penetrate tissues. Nonetheless, conjugation to non-protein polymers, such PEG and the like, is contemplated.

[0206] Modifications other than conjugation are therefore based upon modifying the structure of the antibody fragment to render it more stable, and/or to reduce the rate of catabolism in the body. One mechanism for such modifications is the use of D-amino acids in place of L-amino acids. Those of ordinary skill in the art will understand that the introduction of such modifications needs to be followed by rigorous testing of the resultant molecule to ensure that it still retains the desired biological properties. Further stabilizing modifications include the use of the addition of stabilizing moieties to either N-terminal or C-terminal, or both, which is generally used to prolong half-life of biological molecules. By way of example only, one may wish to modify termini by acylation or amination.

Bispecific Antibodies

[0207] Bispecific antibodies in general may be employed, so long as one arm binds to an aminophospholipid or anionic phospholipid and the bispecific antibody is attached, at a site distinct from the antigen binding site, to a therapeutic agent.

[0208] In general, the preparation of bispecific antibodies is well known in the art. One method involves the separate preparation of antibodies having specificity for the aminophospholipid or anionic phospholipid, on the one hand, and a therapeutic agent on the other. Peptic $F(ab')_2$ fragments are prepared from two chosen antibodies, followed by reduction of each to provide separate Fab'_{SH} fragments. SH groups on one of two partners to be coupled are then alkylated with a cross-linking reagent such as O-phenylenedimaleimide to provide free maleimide groups on one partner. This partner may then be conjugated to the other by means of a thioether linkage, to give the desired $F(ab')_2$ heteroconjugate. Other techniques are known wherein cross-linking with SPDP or protein A is carried out, or a trispecific construct is prepared.

[0209] One method for producing bispecific antibodies is by the fusion of two hybridomas to form a quadroma. As used herein, the term "quadroma" is used to describe the productive fusion of two B cell hybridomas. Using now standard techniques, two antibody producing hybridomas are fused to give daughter cells, and those cells that have maintained the expression of both sets of clonotype immunoglobulin genes are then selected.

CDR Technologies

[0210] Antibodies are comprised of variable and constant regions. The term "variable," as used herein in reference to antibodies, means that certain portions of the variable domains differ extensively in sequence among antibodies, and are used in the binding and specificity of each particular antibody to its particular antigen. However, the variability is concentrated

in three segments termed "hypervariable regions," both in the light chain and the heavy chain variable domains.

[0211] The more highly conserved portions of variable domains are called the framework region (FR). Variable domains of native heavy and light chains each comprise four FRs (FR1, FR2, FR3 and FR4, respectively), largely adopting a beta-sheet configuration connected by three hypervariable regions, which form loops connecting, and in some cases forming part of, the beta-sheet structure.

[0212] The hypervariable regions in each chain are held together in close proximity by the FRs and, with hypervariable regions from the other chain, contribute to the formation of the antigen-binding site of antibodies (Kabat *et al.*, 1991).

Constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

[0213] The term "hypervariable region," as used herein, refers to amino acid residues of an antibody that are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-56 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, .1991) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52(L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101 (H3) in the heavy chain variable domain). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

[0214] The DNA and deduced amino acid sequences of Vh and V kappa chains of the HA antibodies described in Figures 9 and 10 encompass CDR1-3 of variable regions of heavy and light chains of the antibody. In light of the sequence and other information provided herein, and the knowledge in the art, a range of antibodies similar to those described in Figures 9 and 10, and improved antibodies and antigen binding regions, can now be prepared and are thus encompassed by this disclosure. Sequences of the light and heavy chain variable regions of the HA antibodies described in Figures 9 and 10can be determined using standard techniques.

[0215] In certain embodiments, this document provides at least one CDR of the antibody produced by one or more of the HA antibodies described in Figures 9 and 10, to be deposited. In some embodiments, this document provides a CDR, antibody, or antigen binding region thereof, which binds to at least a hemagglutinin, and which comprises at least one CDR of the

antibody produced by one or more of the HA antibodies described in Figures 9 and 10, to be deposited.

[0216] In one embodiment, this document provides an antibody, or antigen binding region thereof, in which the framework regions of one or more of the HA antibodies described in Figures 9 and 10 have been changed from mouse to a human IgG, such as human IgG1 or other IgG subclass to reduce immunogenicity in humans. In some embodiments, sequences of one or more of the HA antibodies described in Figures 9 and 10 are examined for the presence of T-cell epitopes, as is known in the art. The underlying sequence can then be changed to remove T-cell epitopes, *i.e.*, to "deimmunize" the antibody.

[0217] The availability of DNA and amino acid sequences of Vh and V kappa chains of one or more of the HA antibodies described in Figures 9 and 10 means that a range of antibodies can now be prepared using CDR technologies. In particular, random mutations are made in the CDRs and products screened to identify antibodies with higher affinities and/or higher specificities. Such mutagenesis and selection is routinely practiced in the antibody arts, and it can be particularly suitable for use in the methods provided herein, given the advantageous screening techniques disclosed herein. A convenient way for generating such substitutional variants is affinity maturation using phage display.

[0218] CDR shuffling and implantation technologies can be used with antibodies in accordance with the present disclosure, specifically one or more of the HA antibodies described in Figures 9 and 10. CDR shuffling inserts CDR sequences into a specific framework region (Jirholt *et al.*, 1998). CDR implantation techniques permit random combination of CDR sequences into a single master framework (Soderlind *et al.*, 1999, 2000). Using such techniques, CDR sequences of one or more of the HA antibodies described in Figures 9 and 10, for example, are mutagenized to create a plurality of different sequences, which are incorporated into a scaffold sequence and the resultant antibody variants screened for desired characteristics, *e.g.*, higher affinity.

Antibodies from Phagemid Libraries

[0219] Recombinant technology now allows the preparation of antibodies having a desired specificity from recombinant genes encoding a range of antibodies (Van Dijk *et al.*, 1989). Certain recombinant techniques involve isolation of antibody genes by immunological screening of combinatorial immunoglobulin phage expression libraries prepared from RNA isolated from spleen of an immunized animal (Morrison *et al.*, 1986; Winter and Milstein, 1991; Barbas *et al.*, 1992

). For such methods, combinatorial immunoglobulin phagemid libraries are prepared from RNA isolated from spleen of an immunized animal, and phagemids expressing appropriate antibodies are selected by panning using cells expressing antigen and control cells. Advantage of this approach over conventional hybridoma techniques include approximately 10^4 times as many antibodies can be produced and screened in a single round, and that new specificities are generated by H and L chain combination, which further increases the percentage of appropriate antibodies generated.

[0220] One method for the generation of a large repertoire of diverse antibody molecules in bacteria utilizes the bacteriophage lambda as the vector (Huse *et al.*, 1989). Production of antibodies using the lambda vector involves the cloning of heavy and light chain populations of DNA sequences into separate starting vectors. Vectors are subsequently combined randomly to form a single vector that directs co-expression of heavy and light chains to form antibody fragments. The general technique for filamentous phage display is described (U.S. Patent 5,658,727). In a most general sense, the method provides a system for the simultaneous cloning and screening of pre-selected ligand-binding specificities from antibody gene repertoires using a single vector system. Screening of isolated members of the library for a pre-selected ligand-binding capacity allows the correlation of the binding capacity of an expressed antibody molecule with a convenient means to isolate a gene that encodes the member from the library. Additional methods for screening phagemid libraries are described (U.S. Patents 5,580,717; 5,427,908; 5,403,484; and 5,223,409.

[0221] One method for the generation and screening of large libraries of wholly or partially synthetic antibody combining sites, or paratopes, utilizes display vectors derived from filamentous phage such as M13, f1 or fd (U.S. Patent 5,698,426). Filamentous phage display vectors, referred to as "phagemids," yield large libraries of monoclonal antibodies having diverse and novel immunospecificities. The technology uses a filamentous phage coat protein membrane anchor domain as a means for linking gene-product and gene during the assembly stage of filamentous phage replication, and has been used for the cloning and expression of antibodies from combinatorial libraries (Kang *et al.*, 1991; Barbas *et al.*, 1991). The surface expression library is screened for specific Fab fragments that bind hemagglutinin molecules by standard affinity isolation procedures. The selected Fab fragments can be characterized by

sequencing the nucleic acids encoding the polypeptides after amplification of the phage population.

[0222] One method for producing diverse libraries of antibodies and screening for desirable binding specificities is described (U.S. Patents 5,667,988 and 5,759,817). The method involves the preparation of libraries of heterodimeric immunoglobulin molecules in the form of phagemid libraries using degenerate oligonucleotides and primer extension reactions to incorporate degeneracies into CDR regions of immunoglobulin variable heavy and light chain variable domains, and display of mutagenized polypeptides on the surface of the phagemid. Thereafter, the display protein is screened for the ability to bind to a preselected antigen. A further variation of this method for producing diverse libraries of antibodies and screening for desirable binding specificities is described U.S. Patent 5,702,892). In this method, only heavy chain sequences are employed, heavy chain sequences are randomized at all nucleotide positions which encode either the CDRI or CDRIII hypervariable region, and the genetic variability in the CDRs is generated independent of any biological process.

Transgenic Mice Containing Human Antibody Libraries

[0223] Recombinant technology is available for the preparation of antibodies. In addition to the combinatorial immunoglobulin phage expression libraries disclosed above, one molecular cloning approach is to prepare antibodies from transgenic mice containing human antibody libraries. Such techniques are described (U.S. Patent 5,545,807).

[0224] In a most general sense, these methods involve the production of a transgenic animal that has inserted into its germline genetic material that encodes for at least part of an immunoglobulin of human origin or that can rearrange to encode a repertoire of immunoglobulins. The inserted genetic material may be produced from a human source, or may be produced synthetically. The material may code for at least part of a known immunoglobulin or may be modified to code for at least part of an altered immunoglobulin.

[0225] The inserted genetic material is expressed in the transgenic animal, resulting in production of an immunoglobulin derived at least in part from the inserted human immunoglobulin genetic material. The inserted genetic material may be in the form of DNA cloned into prokaryotic vectors such as plasmids and/or cosmids. Larger DNA fragments are inserted using yeast artificial chromosome vectors (Burke *et al.*, 1987), or by introduction of chromosome fragments (Richer *et al.*, 1989). The inserted genetic material may be introduced to the host in

conventional manner, for example by injection or other procedures into fertilized eggs or embryonic stem cells.

[0226] Once a suitable transgenic animal has been prepared, the animal is simply immunized with the desired immunogen. Depending on the nature of the inserted material, the animal may produce a chimeric immunoglobulin, *e.g.* of mixed mouse/human origin, where the genetic material of foreign origin encodes only part of the immunoglobulin; or the animal may produce an entirely foreign immunoglobulin, *e.g.* of wholly human origin, where the genetic material of foreign origin encodes an entire immunoglobulin.

[0227] Polyclonal antisera may be produced from the transgenic animal following immunization. Immunoglobulin-producing cells may be removed from the animal to produce the immunoglobulin of interest. Generally, monoclonal antibodies are produced from the transgenic animal, *e.g.*, by fusing spleen cells from the animal with myeloma cells and screening the resulting hybridomas to select those producing the desired antibody. Suitable techniques for such processes are described herein.

[0228] In one approach, the genetic material may be incorporated in the animal in such a way that the desired antibody is produced in body fluids such as serum or external secretions of the animal, such as milk, colostrum or saliva. For example, by inserting *in vitro* genetic material encoding for at least part of a human immunoglobulin into a gene of a mammal coding for a milk protein and then introducing the gene to a fertilized egg of the mammal, *e.g.*, by injection, the egg may develop into an adult female mammal producing milk containing immunoglobulin derived at least in part from the inserted human immunoglobulin genetic material. The desired antibody can then be harvested from the milk. Suitable techniques for carrying out such processes are known to those skilled in the art.

[0229] The foregoing transgenic animals are usually employed to produce human antibodies of a single isotype, more specifically an isotype that is essential for B cell maturation, such as IgM and possibly IgD. Another method for producing human antibodies is described in U.S. Patents 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and 5,770,429; wherein transgenic animals are described that are capable of switching from an isotype needed for B cell development to other isotypes.

[0230] In the method described in U.S. Patents 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; and 5,770,429, human immunoglobulin transgenes contained within a transgenic animal function correctly throughout the pathway of B-cell development, leading to isotype switching. Accordingly, in this method, these transgenes are constructed so as to produce isotype switching and one or more of the following: (1) high level and cell-type specific

expression, (2) functional gene rearrangement, (3) activation of and response to allelic exclusion, (4) expression of a sufficient primary repertoire, (5) signal transduction, (6) somatic hypermutation, and (7) domination of the transgene antibody locus during the immune response.

Humanized Antibodies

[0231] Human antibodies generally have at least three potential advantages for use in human therapy. First, because the effector portion is human, it may interact better with other parts of the human immune system, *e.g.*, to destroy target cells more efficiently by complement-dependent cytotoxicity (CDC) or antibody-dependent cellular cytotoxicity (ADCC). Second, the human immune system should not recognize the antibody as foreign. Third, half-life in human circulation will be similar to naturally occurring human antibodies, allowing smaller and less frequent doses to be given.

[0232] Various methods for preparing human antibodies are provided herein. In addition to human antibodies, "humanized" antibodies have many advantages. "Humanized" antibodies are generally chimeric or mutant monoclonal antibodies from mouse, rat, hamster, rabbit or other species, bearing human constant and/or variable region domains or specific changes. Techniques for generating a so-called "humanized" antibody are well known to those of skill in the art.

[0233] A number of methods have been described to produce humanized antibodies. Controlled rearrangement of antibody domains joined through protein disulfide bonds to form new, artificial protein molecules or "chimeric" antibodies can be utilized (Konieczny *et al.*, 1981). Recombinant DNA technology can be used to construct gene fusions between DNA sequences encoding mouse antibody variable light and heavy chain domains and human antibody light and heavy chain constant domains (Morrison *et al.*, 1984).

[0234] DNA sequences encoding antigen binding portions or complementarity determining regions (CDR's) of murine monoclonal antibodies can be grafted by molecular means into DNA sequences encoding frameworks of human antibody heavy and light chains (Jones *et al.*, 1986; Riechmann *et al.*, 1988). Expressed recombinant products are called "reshaped" or humanized antibodies, and comprise the framework of a human antibody light or heavy chain and antigen recognition portions, CDR's, of a murine monoclonal antibody.

[0235] One method for producing humanized antibodies is described in U.S. Pat. No. 5,639,641. A similar method for the production of

humanized antibodies is described in U.S. Patents 5,693,762; 5,693,761; 5,585,089; and 5,530,101. These methods involve producing humanized immunoglobulins having one or more complementarity determining regions (CDR's) and possible additional amino acids from a donor immunoglobulin and a framework region from an accepting human immunoglobulin. Each humanized immunoglobulin chain usually comprises, in addition to CDR's, amino acids from the donor immunoglobulin framework that are capable of interacting with CDR's to effect binding affinity, such as one or more amino acids that are immediately adjacent to a CDR in the donor immunoglobulin or those within about 3A as predicted by molecular modeling. Heavy and light chains may each be designed by using any one, any combination, or all of various position criteria described in U.S. Patents 5,693,762; 5,693,761; 5,585,089; and 5,530,101.

When combined into an intact antibody, humanized immunoglobulins are substantially non-immunogenic in humans and retain substantially the same affinity as the donor immunoglobulin to the original antigen.

[0236] An additional method for producing humanized antibodies is described in U.S. Patents 5,565,332 and 5,733,743. This method combines the concept of humanizing antibodies with the phagemid libraries described herein. In a general sense, the method utilizes sequences from the antigen binding site of an antibody or population of antibodies directed against an antigen of interest. Thus for a single rodent antibody, sequences comprising part of the antigen binding site of the antibody may be combined with diverse repertoires of sequences of human antibodies that can, in combination, create a complete antigen binding site.

[0237] Antigen binding sites created by this process differ from those created by CDR grafting, in that only the portion of sequence of the original rodent antibody is likely to make contacts with antigen in a similar manner. Selected human sequences are likely to differ in sequence and make alternative contacts with the antigen from those of the original binding site. However, constraints imposed by binding of the portion of original sequence to antigen and shapes of the antigen and its antigen binding sites, are likely to drive new contacts of human sequences to the same region or epitope of the antigen. This process has therefore been termed "epitope imprinted selection," or "EIS."

[0238] Starting with an animal antibody, one process results in the selection of antibodies that are partly human antibodies. Such antibodies may be sufficiently similar in sequence to human antibodies to be used directly in therapy or after alteration of a few key residues. In EIS, repertoires of antibody fragments can be displayed on the surface of filamentous phase

and genes encoding fragments with antigen binding activities selected by binding of the phage to antigen.

[0239] Yet additional methods for humanizing antibodies contemplated for use are described in U.S. Patents 5,750,078; 5,502,167; 5,705,154; 5,770,403; 5,698,417; 5,693,493; 5,558,864; 4,935,496; and 4,816,567.

[0240] As discussed in the above techniques, the advent of methods of molecular biology and recombinant technology, it is now possible to produce antibodies as described herein by recombinant means and thereby generate gene sequences that code for specific amino acid sequences found in the polypeptide structure of antibodies. This has permitted the ready production of antibodies having sequences characteristic of inhibitory antibodies from different species and sources, as discussed above. In accordance with the foregoing, the antibodies useful in the methods described herein are anti-hemagglutinin antibodies, specifically antibodies whose specificity is toward the same epitope of hemagglutinin as 4F5, 5F5, and 1E11 antibodies described herein, and include all therapeutically active variants and antigen binding fragments thereof whether produced by recombinant methods or by direct synthesis of the antibody polypeptides.

[0241] This document provides plants, plant cells, and plant tissues expressing antibodies that maintain pharmaceutical activity when administered to a subject in need thereof. Exemplary subjects include vertebrates (*e.g.*, mammals, such as humans, and veterinary subjects such as bovines, ovines, canines, and felines). In certain aspects, an edible plant or portion thereof (*e.g.*, sprout, root) can be administered orally to a subject in a therapeutically effective amount. In some aspects one or more influenza antibody is provided in a pharmaceutical preparation, as described herein.

Therapeutic, Prophylactic, and Diagnostic Compositions

[0242] In some embodiments, HA antibodies are used for diagnostic purposes. To give but one example, HA antibodies can be used to identify a subtype, clade, and/or strain of influenza with which a subject is infected. In some embodiments, HA antibodies can be used to identify patient populations that may be responsive to particular influenza treatments.

[0243] This document provides vaccine compositions comprising a least one HA antibody, fusion thereof, and/or portion(s) thereof. In some embodiments, such compositions are intended to elicit a physiological effect upon administration to a subject. A vaccine protein may have healing curative or palliative properties against a disorder or disease and can be administered to ameliorate relieve, alleviate, delay onset of, reverse or lessen symptoms or

severity of a disease or disorder. A vaccine comprising an HA antibody may have prophylactic properties and can be used to prevent or delay the onset of a disease or to lessen the severity of such disease, disorder, or pathological condition when it does emerge. A physiological effect elicited by treatment of a subject with antigen as described herein can include an effective immune response such that infection by an organism is thwarted. Considerations for administration of HA antibodies to a subject in need thereof are discussed in further detail in the section below entitled "*Administration.*"

[0244] In general, active vaccination involves the exposure of a subject's immune system to one or more agents that are recognized as unwanted, undesired, and/or foreign and elicit an endogenous immune response. Typically, such an immune response results in the activation of antigen-specific naive lymphocytes that then give rise to antibody-secreting B cells or antigen-specific effector and memory T cells or both. This approach can result in long-lived protective immunity that may be boosted from time to time by renewed exposure to the same antigenic material.

[0245] In some embodiments, a vaccine composition comprising at least one HA antibody is a subunit vaccine. In general, a subunit vaccine comprises purified antigens rather than whole organisms. Subunit vaccines are not infectious, so they can safely be given to immunosuppressed people, and they are less likely to induce unfavorable immune reactions and/or other adverse side effects. One potential disadvantage of subunit vaccines are that the antigens may not retain their native conformation, so that antibodies produced against the subunit may not recognize the same protein on the pathogen surface; and isolated protein does not stimulate the immune system as well as a whole organism vaccine. Therefore, in some situations, it may be necessary to administer subunit vaccines in higher doses than a whole-agent vaccine (*e.g.*, live attenuated vaccines or inactivated pathogen vaccines) in order to achieve the same therapeutic effect. In contrast, whole-agent vaccines, such as vaccines that utilize live attenuated or inactivated pathogens, typically yield a vigorous immune response, but their use has limitations. For example, live vaccine strains can sometimes cause infectious pathologies, especially when administered to immune-compromised recipients. Moreover, many pathogens, particularly viruses (such as influenza), undergo continuous rapid mutations in their genome, which allow them to escape immune responses to antigenically distinct vaccine strains.

[0246] In some embodiments, subunit vaccines can comprise at least one plant-produced HA antibody. In some embodiments, about 100 μg, about 90 μg, about 80 μg, about 70 μg, about 60 μg, about 50 μg, about 40 μg, about 35 μg, about 30 μg, about 25 μg, about 20 μg, about

15 μg, about 5 μg, about 4 μg, about 3 μg, about 2 μg, or about 1 μg of plant-produced HA antibody and/or immunogenic portion thereof can be used to stimulate an immune response and/or to prevent, delay the onset of, and/or provide protection against influenza infection.

[0247] In some embodiments, this document provides subunit vaccines against influenza. In some embodiments, subunit vaccines comprise an antigen that has been at least partially purified from non-antigenic components. For example, a subunit vaccine may be an HA antibody, fusion thereof, and/or immunogenic portion thereof that is expressed in a live organism (such as a plant, virus, bacterium, yeast, mammalian cell, or egg), but is at least partially purified from the non-antigen components of the live organism. In some embodiments, a subunit vaccine is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or at least 99% purified from the non-antigen components of the organism in which the antigen was expressed. In some embodiments, a subunit vaccine may be an HA antibody, fusion thereof, and/or immunogenic portion thereof that is chemically-synthesized.

[0248] In some embodiments, a subunit vaccine may be an HA antibody, fusion thereof, and/or immunogenic portion thereof that is expressed in a live organism (such as a plant, virus, bacterium, yeast, mammalian cell, or egg), but is *not* at least partially purified from the non-antigen components of the live organism. For example, a subunit vaccine may be an HA antibody, fusion thereof, and/or immunogenic portion thereof that is expressed in a live organism that is administered directly to a subject in order to elicit an immune response. In some embodiments, a subunit vaccine may be an HA antibody, fusion thereof, and/or immunogenic portion thereof that is expressed in a plant, as described herein, wherein the plant material is administered directly to a subject in order to elicit an immune response.

[0249] This document provides pharmaceutical HA antibodies, fusions thereof, and/or immunogenic portions thereof, active as subunit vaccines for therapeutic and/or prophylactic treatment of influenza infection. In certain embodiments, HA antibodies may be produced by plant(s) or portion(s) thereof (*e.g.*, root, cell, sprout, cell line, or plant). In certain embodiments, provided HA antibodies are expressed in plants, plant cells, and/or plant tissues (*e.g.*, sprouts, sprouted seedlings, roots, root culture, clonal cells, clonal cell lines, or clonal plants), and can be used directly from plant or partially purified or purified in preparation for pharmaceutical administration to a subject.

[0250] Also provided are plants, plant cells, and plant tissues expressing HA antibodies that maintain pharmaceutical activity when administered to a subject in need thereof. Exemplary subjects include vertebrates (*e.g.*, mammals such as humans, as well as veterinary subjects

such as bovines, ovines, canines, and felines). In certain aspects, an edible plant or portion thereof (*e.g.*, sprout, root) can be administered orally to a subject in a therapeutically effective amount. In some aspects one or more HA antibodies are provided in a pharmaceutical preparation, as described herein.

[0251] Where it is desirable to formulate an influenza vaccine comprising plant material, it will often be desirable to have utilized a plant that is not toxic to the relevant recipient (*e.g.*, a human or other animal). Relevant plant tissue (*e.g.*, cells, roots, leaves) may simply be harvested and processed according to techniques known in the art, with due consideration to maintaining activity of the expressed product. In certain embodiments, it is desirable to have expressed HA antibodies in an edible plant (and, specifically in edible portions of the plant) so that the material can subsequently be eaten. For instance, where vaccine antigen is active after oral delivery (when properly formulated), it may be desirable to produce antigen protein in an edible plant portion, and to formulate expressed HA antibody for oral delivery together with some or all of the plant material with which the protein was expressed.

[0252] Vaccine compositions can comprise one or more HA antibodies. In certain embodiments, exactly one HA antibody is included in an administered vaccine composition. In certain embodiments, at least two HA antibodies are included in an administered vaccine composition. In some aspects, combination vaccines may include one thermostable fusion protein comprising an HA antibody; in some aspects, two or more thermostable fusion proteins comprising HA antibody are provided.

[0253] In some embodiments, vaccine compositions comprise exactly one HA antibody. In some embodiments, vaccine compositions comprise exactly two HA antibodies. In some embodiments, vaccine compositions comprise exactly three HA antibodies. In some embodiments, vaccine compositions comprise four or more (*e.g.*, 4, 5, 6, 7, 8, 9, 10, 15, or more) HA antibodies.

[0254] In some embodiments, vaccine compositions comprise exactly one HA antibody and exactly one NA antibody (*e.g.*, NA monoclonal antibody 2B9, described in co-pending application U.S.S.N. 11/707,257, filed February 13, 2007, published as US 2008/0124272 on May 29, 2008, entitled "INFLUENZA ANTIBODIES, COMPOSITIONS, AND RELATED METHODS"). In some embodiments, vaccine compositions comprise exactly two HA antibodies and exactly two NA antibodies. In some embodiments, vaccine compositions comprise exactly three HA antibodies and exactly three NA antibodies. In some embodiments, vaccine compositions comprise four or more (*e.g.*, 4, 5, 6, 7, 8, 9, 10, 15, or more) HA antibodies and four or more (*e.g.*, 4, 5, 6, 7, 8, 9, 10, 15, or

more) NA antibodies. In some embodiments, vaccine compositions comprise exactly one HA antibody and two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or more) NA antibodies. In some embodiments, vaccine compositions comprise two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, or more) HA antibodies and exactly one NA antibody.

[0255] In some embodiments, vaccine compositions comprise polytopes (*i.e.*, tandem fusions of two or more amino acid sequences) of two or more HA antibodies and/or immunogenic portions thereof. For example, in some embodiments, a polytope comprises exactly one HA antibody. In some embodiments, a polytope comprises exactly two HA antibodies. In some embodiments, a polytope comprises exactly three HA antibodies. In some embodiments, a polytope comprises four or more (*e.g.*, 4, 5, 6, 7, 8, 9, 10, 15, or more) HA antibodies.

[0256] Where combination vaccines are utilized, it will be understood that any combination of HA antibodies may be used for such combinations. Compositions may include multiple HA antibodies, including multiple antigens provided herein. Furthermore, compositions may include one or more antibodies provided herein with one or more additional antibodies and/or other therapeutic agents. Combinations of HA antibodies include HA antibodies derived from one or more various subtypes or strains such that immunization confers immune response against more than one infection type. Combinations of HA antibodies may include at least one, at least two, at least three, at least four or more antibodies that recognize HA from different influenza subtypes or strains. In some combinations, at least two or at least three antibodies that recognize HA from different influenza subtypes are combined in one vaccine composition.

Additional Vaccine Components

[0257] Vaccine compositions also can include any suitable adjuvant, which can enhance the immunogenicity of the vaccine when administered to a subject. Such adjuvant(s) include, without limitation, saponins, such as extracts of *Quillaja saponaria* (QS), including purified subfractions of food grade QS such as Quil A and QS21; alum; metallic salt particles (*e.g.*, aluminum hydroxide and aluminum phosphate); mineral oil; MF59; Malp2; incomplete Freund's adjuvant; complete Freund's adjuvant; alhydrogel; 3 de-O-acylated monophosphoryl lipid A (3D-MPL); lipid A; *Bortadella pertussis*; *Mycobacterium tuberculosis*; Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); squalene; virosomes; oil-in-water emulsions (*e.g.*, SBAS2); and liposome formulations (*e.g.*, SBAS1). Further adjuvants include immunomodulatory oligonucleotides, for example unmethylated CpG sequences as disclosed in WO 96/02555. Combinations of different adjuvants, such as those mentioned hereinabove, are contemplated as providing an adjuvant which is a

preferential stimulator of TH1 cell response. For example, QS21 can be formulated together with 3D-MPL. The ratio of QS21:3D-MPL will typically be in the order of 1:10 to 10:1; 1:5 to 5:1; and often substantially 1:1. The desired range for optimal synergy may be 2.5:1 to 1:1 3D-MPL: QS21. Doses of purified QS extracts suitable for use in a human vaccine formulation are from 0.01 mg to 10 mg per kilogram of bodyweight.

[0258] It should be noted that certain thermostable proteins (*e.g.*, lichenase) may themselves demonstrate immunoresponse potentiating activity, such that use of such protein whether in a fusion with an HA antibody or separately may be considered use of an adjuvant. Thus, vaccine compositions may further comprise one or more adjuvants. Certain vaccine compositions may comprise two or more adjuvants. Furthermore, depending on formulation and routes of administration, certain adjuvants may be desired in particular formulations and/or combinations.

[0259] In certain situations, it may be desirable to prolong the effect of a vaccine by slowing the absorption of one or more components of the vaccine product (*e.g.*, protein) that is subcutaneously or intramuscularly injected. This may be accomplished by use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of product then depends upon its rate of dissolution, which in turn, may depend upon size and form. Alternatively or additionally, delayed absorption of a parenterally administered product is accomplished by dissolving or suspending the product in an oil vehicle. Injectable depot forms are made by forming microcapsule matrices of protein in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of product to polymer and the nature of the particular polymer employed, rate of release can be controlled. Examples of biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping product in liposomes or microemulsions, which are compatible with body tissues. Alternative polymeric delivery vehicles can be used for oral formulations. For example, biodegradable, biocompatible polymers such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid can be used. Antigen(s) or an immunogenic portions thereof may be formulated as microparticles, *e.g.*, in combination with a polymeric delivery vehicle.

[0260] Enterally administered preparations of vaccine antigens may be introduced in solid, semi-solid, suspension or emulsion form and may be compounded with any pharmaceutically acceptable carriers, such as water, suspending agents, and emulsifying agents. Antigens may be administered by means of pumps or sustained-release forms, especially when administered

as a preventive measure, so as to prevent the development of disease in a subject or to ameliorate or delay an already established disease. Supplementary active compounds, *e.g.*, compounds independently active against the disease or clinical condition to be treated, or compounds that enhance activity of a compound provided herein, can be incorporated into or administered with compositions. Flavorants and coloring agents can be used.

[0261] Vaccine products, optionally together with plant tissue, are particularly well suited for oral administration as pharmaceutical compositions. Oral liquid formulations can be used and may be of particular utility for pediatric populations. Harvested plant material may be processed in any of a variety of ways (*e.g.*, air drying, freeze drying, and/or extraction), depending on the properties of the desired therapeutic product and its desired form. Such compositions as described above may be ingested orally alone or ingested together with food or feed or a beverage. Compositions for oral administration include plants; extractions of plants, and proteins purified from infected plants provided as dry powders, foodstuffs, aqueous or non-aqueous solvents, suspensions, or emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medial parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose or fixed oils. Examples of dry powders include any plant biomass that has been dried, for example, freeze dried, air dried, or spray dried. For example, plants may be air dried by placing them in a commercial air dryer at about 120 °F until biomass contains less than 5% moisture by weight. The dried plants may be stored for further processing as bulk solids or further processed by grinding to a desired mesh sized powder. Alternatively or additionally, freeze-drying may be used for products that are sensitive to air-drying. Products may be freeze dried by placing them into a vacuum drier and dried frozen under a vacuum until the biomass contains less than about 5% moisture by weight. Dried material can be further processed as described herein.

[0262] Plant-derived material may be administered as or together with one or more herbal preparations. Useful herbal preparations include liquid and solid herbal preparations. Some examples of herbal preparations include tinctures, extracts (*e.g.*, aqueous extracts, alcohol extracts), decoctions, dried preparations (*e.g.*, air-dried, spray dried, frozen, or freeze-dried), powders (*e.g.*, lyophilized powder), and liquid. Herbal preparations can be provided in any standard delivery vehicle, such as a capsule, tablet, suppository, or liquid dosage. Those

skilled in the art will appreciate the various formulations and modalities of delivery of herbal preparations that may be applied to the present disclosure.

[0263] Pharmaceutical formulations also can comprise a pharmaceutically acceptable excipient, which, as used herein, includes any and all solvents, dispersion media, diluents, or other liquid vehicles, dispersion or suspension aids, surface active agents, isotonic agents, thickening or emulsifying agents, preservatives, solid binders, lubricants and the like, as suited to the particular dosage form desired. Remington's *The Science and Practice of Pharmacy*, 21st Edition, A. R. Gennaro, (Lippincott, Williams & Wilkins, Baltimore, MD, 2006) discloses various excipients used in formulating pharmaceutical compositions and known techniques for the preparation thereof. Except insofar as any conventional excipient medium is incompatible with a substance or its derivatives, such as by producing any undesirable biological effect or otherwise interacting in a deleterious manner with any other component(s) of the pharmaceutical composition, its use is contemplated to be within the scope of this document.

[0264] In some embodiments, the pharmaceutically acceptable excipient is at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% pure. In some embodiments, the excipient is approved for use in humans and for veterinary use. In some embodiments, the excipient is approved by United States Food and Drug Administration. In some embodiments, the excipient is pharmaceutical grade. In some embodiments, the excipient meets the standards of the United States Pharmacopoeia (USP), the European Pharmacopoeia (EP), the British Pharmacopoeia, and/or the International Pharmacopoeia.

[0265] Pharmaceutically acceptable excipients used in the manufacture of pharmaceutical compositions include, but are not limited to, inert diluents, dispersing and/or granulating agents, surface active agents and/or emulsifiers, disintegrating agents, binding agents, preservatives, buffering agents, lubricating agents, and/or oils. Such excipients may optionally be included in the formulations. Excipients such as cocoa butter and suppository waxes, coloring agents, coating agents, sweetening, flavoring, and/or perfuming agents can be present in the composition, according to the judgment of the formulator.

[0266] Exemplary diluents include, but are not limited to, calcium carbonate, sodium carbonate, calcium phosphate, dicalcium phosphate, calcium sulfate, calcium hydrogen phosphate, sodium phosphate lactose, sucrose, cellulose, microcrystalline cellulose, kaolin, mannitol, sorbitol, inositol, sodium chloride, dry starch, cornstarch, powdered sugar, and/or combinations thereof

[0267] Exemplary granulating and/or dispersing agents include, but are not limited to, potato starch, corn starch, tapioca starch, sodium starch glycolate, clays, alginic acid, guar gum, citrus pulp, agar, bentonite, cellulose and wood products, natural sponge, cation-exchange resins, calcium carbonate, silicates, sodium carbonate, cross-linked poly(vinyl-pyrrolidone) (crospovidone), sodium carboxymethyl starch (sodium starch glycolate), carboxymethyl cellulose, cross-linked sodium carboxymethyl cellulose (croscarmellose), methylcellulose, pregelatinized starch (starch 1500), microcrystalline starch, water insoluble starch, calcium carboxymethyl cellulose, magnesium aluminum silicate (VEEGUM®), sodium lauryl sulfate, quaternary ammonium compounds, and/or combinations thereof.

[0268] Exemplary surface active agents and/or emulsifiers include, but are not limited to, natural emulsifiers (*e.g.*, acacia, agar, alginic acid, sodium alginate, tragacanth, chondrux, cholesterol, xanthan, pectin, gelatin, egg yolk, casein, wool fat, cholesterol, wax, and lecithin), colloidal clays (*e.g.*, bentonite [aluminum silicate] and VEEGUM® [magnesium aluminum silicate]), long chain amino acid derivatives, high molecular weight alcohols (*e.g.*, stearyl alcohol, cetyl alcohol, oleyl alcohol, triacetin monostearate, ethylene glycol distearate, glyceryl monostearate, and propylene glycol monostearate, polyvinyl alcohol), carbomers (*e.g.*, carboxy polymethylene, polyacrylic acid, acrylic acid polymer, and carboxyvinyl polymer), carrageenan, cellulosic derivatives (*e.g.*, carboxymethylcellulose sodium, powdered cellulose, hydroxymethyl cellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, methylcellulose), sorbitan fatty acid esters (*e.g.*, polyoxyethylene sorbitan monolaurate [TWEEN®20], polyoxyethylene sorbitan [TWEEN ®60], polyoxyethylene sorbitan monooleate [TWEEN ®80], sorbitan monopalmitate [SPAN ®40], sorbitan monostearate [SPAN®60], sorbitan tristearate [SPAN ®65], glyceryl monooleate, sorbitan monooleate [SPAN ®80]), polyoxyethylene esters (*e.g.*, polyoxyethylene monostearate [MYRJ®45], polyoxyethylene hydrogenated castor oil, polyethoxylated castor oil, polyoxymethylene stearate, and SOLUTOL®), sucrose fatty acid esters, polyethylene glycol fatty acid esters (*e.g.*, CREMOPHOR®), polyoxyethylene ethers, (*e.g.*, polyoxyethylene lauryl ether [BRIJ®30]), poly(vinyl-pyrrolidone), diethylene glycol monolaurate, triethanolamine oleate, sodium oleate, potassium oleate, ethyl oleate, oleic acid, ethyl laurate, sodium lauryl sulfate, PLURONIC®F 68, POLOXAMER®188, cetrimonium bromide, cetylpyridinium chloride, benzalkonium chloride, docusate sodium, and/or combinations thereof.

[0269] Exemplary binding agents include, without limitation, starch (*e.g.*, cornstarch and starch paste); gelatin; sugars (*e.g.*, sucrose, glucose, dextrose, dextrin, molasses, lactose,

lactitol, and mannitol); natural and synthetic gums [*e.g.*, acacia, sodium alginate, extract of Irish moss, panwar gum, ghatti gum, mucilage of isapol husks, carboxymethylcellulose, methylcellulose, ethylcellulose, hydroxyethylcellulose, hydroxypropyl cellulose, hydroxypropyl methylcellulose, microcrystalline cellulose, cellulose acetate, poly(vinyl-pyrrolidone), magnesium aluminum silicate (VEEGUM®), and larch arabogalactan]; alginates; polyethylene oxide; polyethylene glycol; inorganic calcium salts; silicic acid; polymethacrylates; waxes; water; alcohol; and combinations thereof.

[0270] Exemplary preservatives may include, but are not limited to, antioxidants, chelating agents, antimicrobial preservatives, antifungal preservatives, alcohol preservatives, acidic preservatives, and/or other preservatives. Exemplary antioxidants include, but are not limited to, alpha tocopherol, ascorbic acid, acorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, monothioglycerol, potassium metabisulfite, propionic acid, propyl gallate, sodium ascorbate, sodium bisulfite, sodium metabisulfite, and/or sodium sulfite. Exemplary chelating agents include ethylenediaminetetraacetic acid (EDTA), citric acid monohydrate, disodium edetate, dipotassium edetate, edetic acid, fumaric acid, malic acid, phosphoric acid, sodium edetate, tartaric acid, and/or trisodium edetate. Exemplary antimicrobial preservatives include, but are not limited to, benzalkonium chloride, benzethonium chloride, benzyl alcohol, bronopol, cetrimide, cetylpyridinium chloride, chlorhexidine, chlorobutanol, chlorocresol, chloroxylenol, cresol, ethyl alcohol, glycerin, hexetidine, imidurea, phenol, phenoxyethanol, phenylethyl alcohol, phenylmercuric nitrate, propylene glycol, and/or thimerosal. Exemplary antifungal preservatives include, but are not limited to, butyl paraben, methyl paraben, ethyl paraben, propyl paraben, benzoic acid, hydroxybenzoic acid, potassium benzoate, potassium sorbate, sodium benzoate, sodium propionate, and/or sorbic acid. Exemplary alcohol preservatives include, but are not limited to, ethanol, polyethylene glycol, phenol, phenolic compounds, bisphenol, chlorobutanol, hydroxybenzoate, and/or phenylethyl alcohol. Exemplary acidic preservatives include, but are not limited to, vitamin A, vitamin C, vitamin E, beta-carotene, citric acid, acetic acid, dehydroacetic acid, ascorbic acid, sorbic acid, and/or phytic acid. Other preservatives include, but are not limited to, tocopherol, tocopherol acetate, deteroxime mesylate, cetrimide, butylated hydroxyanisol (BHA), butylated hydroxytoluened (BHT), ethylenediamine, sodium lauryl sulfate (SLS), sodium lauryl ether sulfate (SLES), sodium bisulfite, sodium metabisulfite, potassium sulfite, potassium metabisulfite, GLYDANT PLUS®, PHENONIP®, methylparaben, GERMALL®115, GERMABEN®II, NEOLONE™, KATHON™, and/or EUXYL®.

[0271] Exemplary buffering agents include, but are not limited to, citrate buffer solutions, acetate buffer solutions, phosphate buffer solutions, ammonium chloride, calcium carbonate, calcium chloride, calcium citrate, calcium glubionate, calcium gluceptate, calcium gluconate, D-gluconic acid, calcium glycerophosphate, calcium lactate, propanoic acid, calcium levulinate, pentanoic acid, dibasic calcium phosphate, phosphoric acid, tribasic calcium phosphate, calcium hydroxide phosphate, potassium acetate, potassium chloride, potassium gluconate, potassium mixtures, dibasic potassium phosphate, monobasic potassium phosphate, potassium phosphate mixtures, sodium acetate, sodium bicarbonate, sodium chloride, sodium citrate, sodium lactate, dibasic sodium phosphate, monobasic sodium phosphate, sodium phosphate mixtures, tromethamine, magnesium hydroxide, aluminum hydroxide, alginic acid, pyrogen-free water, isotonic saline, Ringer's solution, ethyl alcohol, and/or combinations thereof.

[0272] Exemplary lubricating agents include, but are not limited to, magnesium stearate, calcium stearate, stearic acid, silica, talc, malt, glyceryl behanate, hydrogenated vegetable oils, polyethylene glycol, sodium benzoate, sodium acetate, sodium chloride, leucine, magnesium lauryl sulfate, sodium lauryl sulfate, and combinations thereof.

[0273] Exemplary oils include, but are not limited to, almond, apricot kernel, avocado, babassu, bergamot, black current seed, borage, cade, camomile, canola, caraway, carnauba, castor, cinnamon, cocoa butter, coconut, cod liver, coffee, corn, cotton seed, emu, eucalyptus, evening primrose, fish, flaxseed, geraniol, gourd, grape seed, hazel nut, hyssop, isopropyl myristate, jojoba, kukui nut, lavandin, lavender, lemon, litsea cubeba, macadamia nut, mallow, mango seed, meadowfoam seed, mink, nutmeg, olive, orange, orange roughy, palm, palm kernel, peach kernel, peanut, poppy seed, pumpkin seed, rapeseed, rice bran, rosemary, safflower, sandalwood, sasquana, savoury, sea buckthorn, sesame, shea butter, silicone, soybean, sunflower, tea tree, thistle, tsubaki, vetiver, walnut, and wheat germ oils. Exemplary oils include, but are not limited to, butyl stearate, caprylic triglyceride, capric triglyceride, cyclomethicone, diethyl sebacate, dimethicone 360, isopropyl myristate, mineral oil, octyldodecanol, oleyl alcohol, silicone oil, and/or combinations thereof.

[0274] Liquid dosage forms for oral and parenteral administration include, but are not limited to, pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and/or elixirs. In addition to active ingredients, liquid dosage forms may comprise inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide,

oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, oral compositions can include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and/or perfuming agents. In certain embodiments for parenteral administration, compositions are mixed with solubilizing agents such a CREMOPHOR®, alcohols, oils, modified oils, glycols, polysorbates, cyclodextrins, polymers, and/or combinations thereof.

[0275] Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing agents, wetting agents, and/or suspending agents. Sterile injectable preparations may be sterile injectable solutions, suspensions, and/or emulsions in nontoxic parenterally acceptable diluents and/or solvents, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P., and isotonic sodium chloride solution. Sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. Fatty acids such as oleic acid can be used in the preparation of injectables.

[0276] Injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter, and/or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[0277] Compositions for rectal or vaginal administration are typically suppositories which can be prepared by mixing compositions with suitable non-irritating excipients such as cocoa butter, polyethylene glycol or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the active ingredient.

[0278] Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the active ingredient is mixed with at least one inert, pharmaceutically acceptable excipient such as sodium citrate or dicalcium phosphate and/or fillers or extenders (*e.g.*, starches, lactose, sucrose, glucose, mannitol, and silicic acid), binders (*e.g.*, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia), humectants (*e.g.*, glycerol), disintegrating agents (*e.g.*, agar, calcium carbonate, potato starch, tapioca starch, alginic acid, certain silicates, and sodium carbonate), solution retarding agents (*e.g.*, paraffin), absorption accelerators (*e.g.*, quaternary ammonium

compounds), wetting agents (*e.g.*, cetyl alcohol and glycerol monostearate), absorbents (*e.g.*, kaolin and bentonite clay), and lubricants (*e.g.*, talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate), and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may comprise buffering agents.

[0279] Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like. The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally comprise opacifying agents and can be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. Solid compositions of a similar type may be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

[0280] Vaccine products, optionally together with plant tissue, are particularly well suited for oral administration as pharmaceutical compositions. Oral liquid formulations can be used and may be of particular utility for pediatric populations. Harvested plant material may be processed in any of a variety of ways (*e.g.*, air drying, freeze drying, and/or extraction), depending on the properties of the desired therapeutic product and its desired form. Such compositions as described above may be ingested orally alone or ingested together with food or feed or a beverage. Compositions for oral administration include plants; extractions of plants, and proteins purified from infected plants provided as dry powders, foodstuffs, aqueous or non-aqueous solvents, suspensions, or emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil, fish oil, and injectable organic esters. Aqueous carriers include water, water-alcohol solutions, emulsions or suspensions, including saline and buffered medial parenteral vehicles including sodium chloride solution, Ringer's dextrose solution, dextrose plus sodium chloride solution, Ringer's solution containing lactose or fixed oils. Examples of dry powders include any plant biomass that has been dried, for example, freeze dried, air dried, or spray dried. For example, plants may be air dried by placing them in a commercial air dryer at about 120°F until biomass contains less than 5% moisture by weight. Dried plants may be stored for further processing as bulk solids or further processed by grinding to a desired mesh sized powder. Alternatively or additionally, freeze-drying may be used for products that are

sensitive to air-drying. Products may be freeze dried by placing them into a vacuum drier and dried frozen under a vacuum until the biomass contains less than about 5% moisture by weight. Dried material can be further processed as described herein.

[0281] Plant-derived material may be administered as or together with one or more herbal preparations. Useful herbal preparations include liquid and solid herbal preparations. Some examples of herbal preparations include tinctures, extracts (*e.g.*, aqueous extracts, alcohol extracts), decoctions, dried preparations (*e.g.*, air-dried, spray dried, frozen, or freeze-dried), powders (*e.g.*, lyophilized powder), and liquid. Herbal preparations can be provided in any standard delivery vehicle, such as a capsule, tablet, suppository, or liquid dosage. Those skilled in the art will appreciate the various formulations and modalities of delivery of herbal preparations that may be applied to the present disclosure.

[0282] In some methods, a plant or portion thereof expressing an HA antibody, or biomass thereof, is administered orally as medicinal food. Such edible compositions can be consumed by eating raw if in a solid form, or by drinking if in liquid form. The plant material can be directly ingested without a prior processing step or after minimal culinary preparation. In some embodiments, a vaccine antigen may be expressed in a sprout that can be eaten directly. For instance, vaccine antigens can be expressed in alfalfa sprouts, mung bean sprouts, spinach leaf sprouts, or lettuce leaf sprouts that can be eaten directly. In some embodiments, plant biomass may be processed and the material recovered after the processing step can be ingested.

[0283] Processing methods useful in accordance with the present disclosure are methods commonly used in the food or feed industry. Final products of such methods typically include a substantial amount of an expressed antigen and can be conveniently eaten or drunk. The final product may be mixed with other food or feed forms, such as salts, carriers, favor enhancers, antibiotics, and the like, and consumed in solid, semi-solid, suspension, emulsion, or liquid form. Such methods can include a conservation step, such as, *e.g.*, pasteurization, cooking, or addition of conservation and preservation agents. Any plant may be used and processed to produce edible or drinkable plant matter. The amount of HA antibody in a plant-derived preparation may be tested by methods standard in the art, *e.g.*, gel electrophoresis, ELISA, or western blot analysis, using a probe or antibody specific for product. This determination may be used to standardize the amount of vaccine antigen protein ingested. For example, the amount of vaccine antigen may be determined and regulated, for example, by mixing batches of product having different levels of product so that the quantity of material to be drunk or eaten to ingest a single dose can be standardized.

A contained, regulatable environment should, however, minimize the need to carry out such standardization procedures.

[0284] A vaccine protein produced in a plant cell or tissue and eaten by a subject may be preferably absorbed by the digestive system. One advantage of the ingestion of plant tissue that has been only minimally processed is to provide encapsulation or sequestration of the protein in cells of the plant. Thus, product may receive at least some protection from digestion in the upper digestive tract before reaching the gut or intestine and a higher proportion of active product would be available for uptake.

[0285] Dosage forms for topical and/or transdermal administration of a compound as provided herein may include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants and/or patches. Generally, the active ingredient is admixed under sterile conditions with a pharmaceutically acceptable excipient and/or any needed preservatives and/or buffers as may be required. Additionally, this disclosure contemplates the use of transdermal patches, which often have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms may be prepared, for example, by dissolving and/or dispensing the compound in the proper medium. Alternatively or additionally, the rate may be controlled by either providing a rate controlling membrane and/or by dispersing the compound in a polymer matrix and/or gel.

[0286] Suitable devices for use in delivering intradermal pharmaceutical compositions described herein include short needle devices such as those described in U.S. Patents 4,886,499; 5,190,521; 5,328,483; 5,527,288; 4,270,537; 5,015,235; 5,141,496; and 5,417,662. Intradermal compositions may be administered by devices which limit the effective penetration length of a needle into the skin, such as those described in PCT publication WO 99/34850 and functional equivalents thereof. Jet injection devices which deliver liquid vaccines to the dermis via a liquid jet injector and/or via a needle which pierces the stratum corneum and produces a jet which reaches the dermis are suitable. Jet injection devices are described, for example, in U.S. Patents 5,480,381; 5,599,302; 5,334,144; 5,993,412; 5,649,912; 5,569,189; 5,704,911; 5,383,851; 5,893,397; 5,466,220; 5,339,163; 5,312,335; 5,503,627; 5,064,413; 5,520,639; 4,596,556; 4,790,824; 4,941,880; 4,940,460; and PCT publications WO 97/37705 and WO 97/13537. Ballistic powder/particle delivery devices which use compressed gas to accelerate vaccine in powder form through the outer layers of the skin to the dermis are suitable. Alternatively or additionally, conventional syringes may be used in the classical mantoux method of intradermal administration.

[0287] Formulations suitable for topical administration include, but are not limited to, liquid and/or semi liquid preparations such as liniments, lotions, oil in water and/or water in oil emulsions such as creams, ointments and/or pastes, and/or solutions and/or suspensions. Topically administrable formulations may, for example, comprise from about 1% to about 10% (w/w) active ingredient, although the concentration of the active ingredient may be as high as the solubility limit of the active ingredient in the solvent. Formulations for topical administration may further comprise one or more of the additional ingredients described herein.

[0288] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for pulmonary administration via the buccal cavity. Such a formulation may comprise dry particles which comprise the active ingredient and which have a diameter in the range from about 0.5 nm to about 7 nm or from about 1 nm to about 6 nm. Such compositions are conveniently in the form of dry powders for administration using a device comprising a dry powder reservoir to which a stream of propellant may be directed to disperse the powder and/or using a self propelling solvent/powder dispensing container such as a device comprising the active ingredient dissolved and/or suspended in a low-boiling propellant in a sealed container. Such powders comprise particles wherein at least 98% of the particles by weight have a diameter greater than 0.5 nm and at least 95% of the particles by number have a diameter less than 7 nm. Alternatively, at least 95% of the particles by weight have a diameter greater than 1 nm and at least 90% of the particles by number have a diameter less than 6 nm. Dry powder compositions may include a solid fine powder diluent such as sugar and are conveniently provided in a unit dose form.

[0289] Low boiling propellants generally include liquid propellants having a boiling point of below 65 °F at atmospheric pressure. Generally the propellant may constitute 50% to 99.9% (w/w) of the composition, and the active ingredient may constitute 0.1% to 20% (w/w) of the composition. The propellant may further comprise additional ingredients such as a liquid non-ionic and/or solid anionic surfactant and/or a solid diluent (which may have a particle size of the same order as particles comprising the active ingredient).

[0290] Pharmaceutical compositions formulated for pulmonary delivery may provide the active ingredient in the form of droplets of a solution and/or suspension. Such formulations may be prepared, packaged, and/or sold as aqueous and/or dilute alcoholic solutions and/or suspensions, optionally sterile, comprising the active ingredient, and may conveniently be administered using any nebulization and/or atomization device. Such formulations may further comprise one or more additional ingredients including, but not limited to, a flavoring

agent such as saccharin sodium, a volatile oil, a buffering agent, a surface-active agent, and/or a preservative such as methylhydroxybenzoate. The droplets provided by this route of administration may have an average diameter in the range from about 0.1 nm to about 200 nm.

[0291] Formulations described herein as being useful for pulmonary delivery are useful for intranasal delivery of a pharmaceutical composition. Another formulation suitable for intranasal administration is a coarse powder comprising the active ingredient and having an average particle from about 0.2 μm to 500 μm. Such a formulation is administered in the manner in which snuff is taken, *i.e.*, by rapid inhalation through the nasal passage from a container of the powder held close to the nose.

[0292] Formulations suitable for nasal administration may, for example, comprise from about as little as 0.1% (w/w) and as much as 100% (w/w) of the active ingredient, and may comprise one or more of the additional ingredients described herein. A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for buccal administration. Such formulations may, for example, be in the form of tablets and/or lozenges made using conventional methods, and may, for example, 0.1% to 20% (w/w) active ingredient, the balance comprising an orally dissolvable and/or degradable composition and, optionally, one or more of the additional ingredients described herein. Alternately, formulations suitable for buccal administration may comprise a powder and/or an aerosolized and/or atomized solution and/or suspension comprising the active ingredient. Such powdered, aerosolized, and/or aerosolized formulations, when dispersed, may have an average particle and/or droplet size in the range from about 0.1 nm to about 200 nm, and may further comprise one or more of the additional ingredients described herein.

[0293] A pharmaceutical composition may be prepared, packaged, and/or sold in a formulation suitable for ophthalmic administration. Such formulations may, for example, be in the form of eye drops including, for example, a 0.1/1.0% (w/w) solution and/or suspension of the active ingredient in an aqueous or oily liquid excipient. Such drops may further comprise buffering agents, salts, and/or one or more other of the additional ingredients described herein. Other opthalmically-administrable formulations which are useful include those which comprise the active ingredient in microcrystalline form and/or in a liposomal preparation. Ear drops and/or eye drops are contemplated as being within the scope of this document.

[0294] In certain situations, it may be desirable to prolong the effect of a vaccine by slowing the absorption of one or more components of the vaccine product (*e.g.*, protein) that is

subcutaneously or intramuscularly injected. This may be accomplished by use of a liquid suspension of crystalline or amorphous material with poor water solubility. The rate of absorption of product then depends upon its rate of dissolution, which in turn, may depend upon size and form. Alternatively or additionally, delayed absorption of a parenterally administered product is accomplished by dissolving or suspending the product in an oil vehicle. Injectable depot forms are made by forming microcapsule matrices of protein in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of product to polymer and the nature of the particular polymer employed, rate of release can be controlled. Examples of biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations may be prepared by entrapping product in liposomes or microemulsions, which are compatible with body tissues. Alternative polymeric delivery vehicles can be used for oral formulations. For example, biodegradable, biocompatible polymers such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid, can be used. Antigen(s) or an immunogenic portions thereof may be formulated as microparticles, *e.g.*, in combination with a polymeric delivery vehicle.

[0295] General considerations in the formulation and/or manufacture of pharmaceutical agents may be found, for example, in *Remington: The Science and Practice of Pharmacy* 21st ed., Lippincott Williams & Wilkins, 2005.

Administration

[0296] Among other things, this document provides subunit vaccines. In some embodiments, subunit vaccines may be administered to a subject at low doses in order to stimulate an immune response and/or confer protectivity. As used herein, the term "low-dose vaccine" generally refers to a vaccine that is immunogenic and/or protective when administered to a subject at low-doses. Administration of a low-dose vaccine can comprise administration of a subunit vaccine composition comprising less than 100 μg of an HA antibody, fusion thereof, and/or immunogenic portion thereof.

[0297] In some embodiments, administration of a low-dose subunit vaccine comprises administering a subunit vaccine comprising less than about 100 μg, less than about 90 μg, less than about 80 μg, less than about 70 μg, less than about 60 μg, less than about 50 μg, less than about 40 μg, less than about 35 μg, less than about 30 μg, less than about 25 μg, less than about 20 μg, less than about 15 μg, less than about 5 μg, less than about 4 μg, less than about 3 μg, less than about 2 μg, or less than about 1 μg of plant-produced HA antibody,

fusion thereof, and/or immunogenic portion thereof to a subject in need thereof. In some embodiments, the plant-produced HA antibody, fusion thereof, and/or immunogenic portion thereof has been at least partially purified from non-antigenic components, as described herein. In some embodiments, the plant-produced HA antibody, fusion thereof, and/or immunogenic portion thereof has *not* been at least partially purified from non-antigenic components, as described herein. Suitable vaccine compositions for administration to a subject are described in further detail in the section above, entitled "*Vaccines*."

[0298] HA antibodies, fusions thereof, and/or immunogenic portions thereof, and/or pharmaceutical compositions thereof (*e.g.*, vaccines) may be administered using any amount and any route of administration effective for treatment.

[0299] The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the infection, the particular composition, its mode of administration, its mode of activity, and the like. HA antibodies are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions provided herein will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject or organism will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific HA antibody employed; the specific pharmaceutical composition administered; the half-life of the composition after administration; the age, body weight, general health, sex, and diet of the subject; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors, well known in the medical arts.

[0300] Pharmaceutical compositions (*e.g.*, vaccines) may be administered by any route. In some embodiments, pharmaceutical compositions can be administered by a variety of routes, including oral (PO), intravenous (IV), intramuscular (IM), intra-arterial, intramedullary, intrathecal, subcutaneous (SQ), intraventricular, transdermal, interdermal, intradermal, rectal (PR), vaginal, intraperitoneal (IP), intragastric (IG), topical (*e.g.*, by powders, ointments, creams, gels, lotions, and/or drops), mucosal, intranasal, buccal, enteral, vitreal, sublingual; by intratracheal instillation, bronchial instillation, and/or inhalation; as an oral spray, nasal spray, and/or aerosol; and/or through a portal vein catheter. In general, the most appropriate route of administration will depend upon a variety of factors, including the nature of the agent being administered (*e.g.*, its stability in the environment of the gastrointestinal tract) and the

condition of the subject (*e.g.*, whether the subject is able to tolerate a particular mode of administration).

[0301] In some embodiments, vaccines are delivered by multiple routes of administration (*e.g.*, by subcutaneous injection and by intranasal inhalation). For vaccines involving two or more doses, different doses may be administered via different routes.

[0302] In some embodiments, vaccines are delivered by subcutaneous injection. In some embodiments, vaccines are administered by intramuscular and/or intravenous injection. In some embodiments, vaccines are delivered by intranasal inhalation.

[0303] In some embodiments, vaccines as provided herein are delivered by oral and/or mucosal routes. Oral and/or mucosal delivery has the potential to prevent infection of mucosal tissues, the primary gateway of infection for many pathogens. Oral and/or mucosal delivery can prime systemic immune response. There has been considerable progress in the development of heterologous expression systems for oral administration of antigens that stimulate the mucosal-immune system and can prime systemic immunity. Previous efforts at delivery of oral vaccine however, have demonstrated a requirement for considerable quantities of antigen in achieving efficacy. Thus, economical production of large quantities of target antigens is a prerequisite for creation of effective oral vaccines. Development of plants expressing antigens, including thermostable antigens, represents a more realistic approach to such difficulties.

[0304] In certain embodiments, an HA antibody expressed in a plant or portion thereof is administered to a subject orally by direct administration of a plant to a subject. In some aspects a vaccine protein expressed in a plant or portion thereof is extracted and/or purified, and used for the preparation of a pharmaceutical composition. It may be desirable to formulate such isolated products for their intended use (*e.g.*, as a pharmaceutical agent or vaccine composition). In some embodiments, it will be desirable to formulate products together with some or all of plant tissues that express them.

[0305] In certain embodiments, an HA antibody expressed in a plant or portion thereof is administered to a subject orally by direct administration of a plant to a subject. In some aspects a vaccine protein expressed in a plant or portion thereof is extracted and/or purified, and used for preparation of a pharmaceutical composition. It may be desirable to formulate such isolated products for their intended use (*e.g.*, as a pharmaceutical agent or vaccine composition). In some embodiments, it will be desirable to formulate products together with some or all of plant tissues that express them.

[0306] A vaccine protein produced in a plant cell or tissue and eaten by a subject may be preferably absorbed by the digestive system. One advantage of the ingestion of plant tissue that has been only minimally processed is to provide encapsulation or sequestration of the protein in cells of the plant. Thus, product may receive at least some protection from digestion in the upper digestive tract before reaching the gut or intestine and a higher proportion of active product would be available for uptake.

[0307] Where it is desirable to formulate product together with plant material, it will often be desirable to have utilized a plant that is not toxic to the relevant recipient (*e.g.*, a human or other animal). Relevant plant tissue (*e.g.*, cells, roots, leaves) may simply be harvested and processed according to techniques known in the art, with due consideration to maintaining activity of the expressed product. In certain embodiments, it is desirable to have expressed HA antibody in an edible plant (and, specifically in edible portions of the plant) so that the material can subsequently be eaten. For instance, where vaccine antigen is active after oral delivery (when properly formulated), it may be desirable to produce antigen protein in an edible plant portion, and to formulate expressed HA antibody for oral delivery together with some or all of the plant material with which a protein was expressed.

[0308] In certain embodiments, HA antibodies as provided herein and/or pharmaceutical compositions thereof (*e.g.*, vaccines) may be administered at dosage levels sufficient to deliver from about 0.001 mg/kg to about 100 mg/kg, from about 0.01 mg/kg to about 50 mg/kg, from about 0.1 mg/kg to about 40 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 0.01 mg/kg to about 10 mg/kg, from about 0.1 mg/kg to about 10 mg/kg, or from about 1 mg/kg to about 25 mg/kg of subject body weight per day to obtain the desired therapeutic effect. The desired dosage may be delivered more than three times per day, three times per day, two times per day, once per day, every other day, every third day, every week, every two weeks, every three weeks, every four weeks, every two months, every six months, or every twelve months. In certain embodiments, the desired dosage may be delivered using multiple administrations (*e.g.*, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, or more administrations).

[0309] Compositions are administered in such amounts and for such time as is necessary to achieve the desired result. In certain embodiments, a "therapeutically effective amount" of a pharmaceutical composition is that amount effective for treating, attenuating, or preventing a disease in a subject. Thus, the "amount effective to treat, attenuate, or prevent disease," as used herein, refers to a nontoxic but sufficient amount of the pharmaceutical composition to

treat, attenuate, or prevent disease in any subject. For example, the "therapeutically effective amount" can be an amount to treat, attenuate, or prevent infection (*e.g.*, influenza infection)

[0310] It will be appreciated that HA antibodies and/or pharmaceutical compositions thereof can be employed in combination therapies. The particular combination of therapies (*e.g.*, therapeutics or procedures) to employ in a combination regimen will take into account compatibility of the desired therapeutics and/or procedures and the desired therapeutic effect to be achieved. It will be appreciated that the therapies employed may achieve a desired effect for the same purpose (for example, HA antibodies useful for treating, preventing, and/or delaying the onset of influenza infection may be administered concurrently with another agent useful for treating, preventing, and/or delaying the onset of influenza infection), or they may achieve different effects (*e.g.*, control of any adverse effects). This document encompasses the delivery of pharmaceutical compositions in combination with agents that may improve their bioavailability, reduce and/or modify their metabolism, inhibit their excretion, and/or modify their distribution within the body.

[0311] Pharmaceutical compositions in accordance with the present disclosure may be administered either alone or in combination with one or more other therapeutic agents. By "in combination with," it is not intended to imply that the agents must be administered at the same time and/or formulated for delivery together, although these methods of delivery are within the scope of this document. Compositions can be administered concurrently with, prior to, or subsequent to, one or more other desired therapeutics or medical procedures. In will be appreciated that therapeutically active agents utilized in combination may be administered together in a single composition or administered separately in different compositions. In general, each agent will be administered at a dose and/or on a time schedule determined for that agent.

[0312] In general, it is expected that agents utilized in combination with be utilized at levels that do not exceed the levels at which they are utilized individually. In some embodiments, the levels utilized in combination will be lower than those utilized individually.

[0313] In certain embodiments, vaccine compositions comprising at least one HA antibody are administered in combination with other influenza vaccines. In certain embodiments, vaccine compositions comprising at least one HA antibody are administered in combination with other influenza therapeutics. In certain embodiments, vaccine compositions comprising at least one HA antibody are administered in combination with antiviral drugs, such as neuraminidase inhibitors (*e.g.*, oseltamivir [TAMIFLU®], zanamivir [RELENZAAND®] and/or M2 inhibitors (*e.g.*, adamantane, adamantane derivatives, and rimantadine).

Kits

[0314] In one aspect, this document provides a pharmaceutical pack or kit including at least one HA antibody as provided herein. In certain embodiments, pharmaceutical packs or kits include live sprouted seedlings, clonal entity or plant producing an antibody or antigen binding fragment as provided herein, or preparations, extracts, or pharmaceutical compositions containing antibody in one or more containers filled with optionally one or more additional ingredients of pharmaceutical compositions as provided herein. In some embodiments, pharmaceutical packs or kits include pharmaceutical compositions comprising purified HA antibody, in one or more containers optionally filled with one or more additional ingredients of pharmaceutical compositions. In certain embodiments, the pharmaceutical pack or kit includes an additional approved therapeutic agent (*e.g.*, influenza antibody, influenza vaccine, influenza therapeutic) for use as a combination therapy. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceutical products, which notice reflects approval by the agency of manufacture, use, or sale for human administration.

[0315] Kits are provided that include therapeutic reagents. As but one non-limiting example, HA antibody can be provided as oral formulations and administered as therapy. Alternatively or additionally, HA antibody can be provided in an injectable formulation for administration. In one embodiment, HA antibody can be provided in an inhalable formulation for administration. Pharmaceutical doses or instructions therefore may be provided in the kit for administration to an individual suffering from or at risk for influenza infection.

[0316] The representative examples that follow are intended to help illustrate the invention, and are not intended to, nor should they be construed to, limit the scope of the invention. Indeed, various modifications of the invention and many further embodiments thereof, in addition to those shown and described herein, will become apparent to those skilled in the art from the full contents of this document, including the examples which follow and the references to the scientific and patent literature cited herein. The following examples contain information, exemplification and guidance, which can be adapted to the practice of this invention in its various embodiments and the equivalents thereof.

EXAMPLES

Example 1. Cloning, expression, and purification of influenza HA

[0317] The HA sequences encoding hemagglutinin (HA) from A/Anhui/1/2005 (SEQ ID NO:22), A/Bar-headed goose/Qinghai/0510/05 (SEQ ID NO:24), A/Indonesia/5/05 (SEQ ID NO:23), and A/Wyoming/3/03 (H3N2) (SEQ ID NO:34) were optimized for expression in plants and synthesized by GENEART AG (Regensburg, Germany). The PR-1a signal peptide was added to the N-terminus and the endoplasmic reticulum retention signal (KDEL) and a poly-histidine affinity purification tag (His_6) were added to the C-terminus. The resulting sequence was inserted into the launch vector pGRD4 to obtain pGRD4-HA (illustrated in Figure 1). The pGRD4 vector is based on *Tobacco mosaic virus* (TMV) and was engineered using the pGreen/pSoup system as a binary expression vector by introducing the *Cauliflower mosaic virus* (CaMV) 35S promoter, the *nos* terminator, and the hammerhead ribozyme sequence from the launch vector pBID4. pGRD4-HA and pSoup, which provides replication functions in trans, were then introduced into *Agrobacterium tumefaciens* strain GV3101. The resulting bacterial strain was grown in AB medium (18.7 mM NH_4Cl, 2.5 mM $MgSO_4$, 2 mM KCl, 0.07 mM $CaCl_2$, 2.7 μM $FeSO_4$, 17.2 mM K_2HPO_4, 6.4 mM NaH_2PO_4, 0.2% glucose) overnight at 28°C. The bacteria were introduced into the aerial parts of 6-week-old *Nicotiana benthamiana* plants grown hydroponically in rockwool slabs, by vacuum infiltration at a cell density of OD_{600} = 0.5. Seven days after vacuum infiltration, leaf tissue was harvested, and homogenized using a household blender. The extracts were clarified by centrifugation (78 000 x *g* for 30 min) and HA was purified using Ni-column chromatography (pre-packed His Trap HP Ni columns, GE Healthcare, NJ). Further purification was carried out by anion exchange chromatography (Sepharose Q columns, GE Healthcare, NJ) on a Bio-Rad Duo Flow system using Biologics software.

Example 2: Generation of Plants and Antigen Production

[0318] Agrobacterium Infiltration of Plants: Agrobacterium-mediated transient expression system achieved by Agrobacterium infiltration was utilized (Turpen *et al.* (1993) J. Virol. Methods 42:227). Healthy leaves of *Nicotiana benthamiana* were infiltrated with A. rhizogenes containing viral vectors engineered to express N1NA.

[0319] The *A. tumifaciens* strain A4 (ATCC 43057; ATCC, Manassas, VA) was transformed with the constructs pBI-D4-PR-NA-KDEL and pBI-D4-PR-NA-VAC. Agrobacterium cultures were grown and induced as described (Kapila *et al.* (1997) Plant Sci. 122:101). A 2 ml starter-culture (picked from a fresh colony) was grown overnight in YEB (5 g/l beef

extract, 1 g/l yeast extract, 5 g/l peptone, 5 g/l sucrose, 2 mM MgSO4) with 25 μg/ml kanamycin at 28°C. The starter culture was diluted 1:500 into 500 ml of YEB with 25 μg/ml kanamycin, 10 mM 2-4(-morpholino)ethanesulfonic acid (MES) pH 5.6, 2 mM additional MgSO4 and 20 μM acetosyringone. The diluted culture was then grown overnight to an O.D.600 of ~1.7 at 28°C. The cells were centrifuged at 3,000 x g for 15 minutes and re-suspended in MMA medium (MS salts, 10 mM MES pH 5.6, 20 g/l sucrose, 200 μM acetosyringone) to an O.D.600 of 2.4, kept for 1-3 hour at room temperature, and used for Agrobacterium-infiltration. *N. benthamiana* leaves were injected with the Agrobacterium-suspension using a disposable syringe without a needle. Infiltrated leaves were harvested 6 days post-infiltration. Plants were screened for the presence of target antigen expression by immunoblot analysis.

Example 3: Production of Antigen

[0320] 100 mg samples of *N. benthamiana* infiltrated leaf material were harvested at 4, 5, 6 and 7 days post-infection. The fresh tissue was analyzed for protein expression right after being harvested or collected at -80°C for the preparation of subsequent crude plants extracts or for fusion protein purification.

[0321] Fresh samples were resuspended in cold PBS 1x plus protease inhibitors (Roche) in a 1/3 w/v ratio (1 ml / 0.3 g of tissue) and ground with a pestle. The homogenates were boiled for 5 minutes in SDS gel loading buffer and then clarified by centrifugation for 5 minutes at 12,000 rpm at 4°C. The supernatants were transferred to fresh tubes, and 20 μl, 1 μl, or dilutions thereof were separated by 12% SDS-PAGE and analyzed by Western analysis using anti-His6-HA mouse polyclonal antibodies.

[0322] HA expression in *N. benthamiana* plants infiltrated either with *A. tumefaciens* containing the plasmid pBID4-HA-KDEL led to a specific band corresponding to the molecular weight of NA-KDEL. Quantification of HA-KDEL expressed in crude extract was made by immunoblotting both on manually infiltrated tissues and on vacuum-infiltrated tissues.

[0323] *Purification of Antigens*: Leaves from plants infiltrated with recombinant A. tumefaciens containing the pBID4-HA-KDEL construct were ground by homogenization. Extraction buffer with "EDTA-free" protease inhibitors (Roche) and 1% Triton™ X-100 was used at a ratio of 3x (w/v) and rocked for 30 minutes at 4°C. Extracts were clarified by centrifugation at 9000 x g for 10 minutes at 4°C. Supernatants were sequentially filtered

through Mira cloth, centrifuged at 20,000 x g for 30 minutes at 4°C, and filtered through a 0.45-μm filter before chromatographic purification.

[0324] The resulting extracts were cut using ammonium sulfate precipitation. Briefly, $(NH_4)_2SO_4$ was added to 20% saturation, incubated on ice for 1 hour, and spun down at 18,000 x g for 15 minutes. Pellets were discarded and $(NH_4)_2SO_4$ was added slowly to 60% saturation, incubated on ice for 1 hour, and spun down at 18,000 x g for 15 minutes. Supernatants were discarded and the resulting pellets were resuspended in buffer, maintained on ice for 20 minutes, and centrifuged at 18,000 x g for 30 minutes. Supernatants were dialyzed overnight against 10,000 volumes of washing buffer.

[0325] His-tagged HA-KDEL proteins were purified using Ni-NTA sepharose ("Chelating Sepharose Fast Flow Column;" Amersham) at room temperature under gravity. The purification was performed under non-denaturing conditions. Proteins were collected as 0.5 ml fractions that were unified, combined with 20 mM EDTA, dialyzed against 1x PBS overnight at 4°C, and analyzed by SDS-PAGE. Alternatively, fractions were collected, unified, combined with 20 mM EDTA, dialyzed against 10mM NaH2PO4 overnight at 4°C, and purified by anion exchange chromatography. For HA-KDEL purification, anion exchange column Q Sepharose Fast Flow (Amersham Pharmacia Biosciences) was used. Samples of the HA-KDEL affinity or ion-exchange purified proteins were separated on 12% polyacrylamide gels followed by Coomassie staining.

[0326] After dialysis, samples were analyzed by immunoblotting using the mAb α-anti-His6. The His-tag was maintained by the expressed proteins, and the final concentration of the purified protein was determined using GeneTools software from Syngene (Frederick, MD).

Example 4: Western blot and ELISA analysis of purified ppH5HA-I

[0327] Samples of HA, purified from infiltrated *N. benthamiana* leaves, were separated on 10% SDS-polyacrylamide gels, transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA) and blocked with 0.5% I-block (Applied Biosystems, CA). The membrane was then incubated with a mouse monoclonal antibody against poly-His (Roche-Applied-Science, IN) followed by horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratory Inc., PA). Proteins reacting with the anti-His antibody were visualized using SuperSignal West Pico Chemiluminescent Substrate (Pierce, IL). The image was taken using GeneSnap software on a GeneGnome and quantified using Gene Tools software (Syngene Bioimaging, MD).

Example 5: Derivation of a Murine Hybridoma Secreting Monoclonal Antibody

[0328] Six-week-old Balb/c mice were immunized with plant-produced HA subcutaneously at 2-week intervals on days 0, 14, and 28. All immunizations included 10 μg of Quil A adjuvant (Accurate Chemical, NY). Mice were boosted intraperitoneally 3-4 days prior to spleen harvest. Spleens were teased into single cell suspensions and red blood cells were lysed (NH_4Cl solution). Splenocytes were combined with P3 myeloma cells at a 1:1 ratio. A solution of 50% PEG was added and the cells were incubated at 37°C for 2 hrs. After the incubation, the cell pellet was resuspended in media containing 1x HAT and plated in 96 well plates and incubated for two weeks. HAT containing media was replaced as needed. On day 14, the cells were feed media containing HT (not HAT) for 1 week, replacing media every-other day. At this point the hybridoma cell lines were screened by ELISA, initially on plant-produced antigen and then inactivated virus. Positive wells were put through several rounds of limiting dilution to isolate a single clone and screened again. At this point, the isotype of the antibody was determined and cell supernatants were screened for functionality by hemagglutination inhibition assays (HI) against homologous and heterologous viruses. Antibodies from selected clones were then purified from mouse ascites at Rockland Immunochemicals.

[0329] 45 million spleen cells were fused with 5 million P3XAg8.653 murine myeloma cells using polyethylene glycol. The resulting 50 million fused cells were plated at 5 X 10^5 cells per well in 10 X 96 well plates. HAT (hypoxanthine, aminopterin, and thymidine) selection followed 24 hours later and continued until colonies arose. All immunoglobulin-secreting hybridomas were subcloned by three rounds of limiting dilution in the presence of HAT.

[0330] Hybridomas were screened on ELISA plates for secretion of H5 HA specific immunoglobulin. Hybridomas 1E11, 4F5, 5F5, 13B8, 1E5 and 2C7 each had a high specific signal.

Example 6: Characterization of mAb specificity

[0331] The specificity of each mAb to homologous and heterologous influenza viruses was analyzed by ELISA. Plates were coated with inactivated H5N1 or H3N2 virus; antibody concentration was 125 ng/ml. The reactivity of mAb 4F5 (A/Anhui/1/05), mAb5F5 (A/Bar-headed goose/Qinghai/1A/05 and mAb 1E11 (A/Bar-headed goose/Qinghai/1A/05) against a panel of influenza strains (iA Vietnam (H5N1); iA/Indonesia (H5N1); A/Anhui/1/05 (H5N1)

; A/Bar-headed goose/Qinghai/1A/05 (H5N1); and iA/Wyoming (H3N2)) is shown in Figure 2A, Figure 2B and Figure 2C, respectively.

Example 7: Hemagglutination Inhibition Activity of mAbs 1E11, 5F5 and 4F5

[0332] MAbs 1E11, 5F5 and 4F5 were assayed for hemagglutination inhibition activity. Culture supernatants of hybridoma cells were treated with receptor-destroying enzyme (RDE; Denka Seiken Co. Ltd., Tokyo, Japan) and an HI assay was carried out with 1% horse erythrocytes containing 0.1% bovine serum albumin, as described in Noah *et al.* (Clin Vaccine Immunol.,16(4), 558-566 2009). Briefly, two-fold serial dilution of culture supernatant or purified monoclonal antibodies were mixed with 8 HAU/50 µl of influenza virus in the V-bottom 96-well plates and incubated for 45 to 60 minutes at room temperature. Horse erythrocytes were diluted to 1% with PBS and then added to the 96-well plates containing antibody/serum mixture. After 30-45 minutes incubation, wells were observed for agglutination and the HI titer of the individual samples was determined as the reciprocal of the highest dilution which caused complete inhibition of hemagglutination. Sheep anti-A/Vietnam/1194/04 was used as a reference serum.

[0333] The results of this experiment are shown in Figure 3. The values in the table are the lowest concentration (ug/ml) that inhibited hemagglutination activity (8HAU/50 ul) for each strain. For the reference serum, the numbers shown are endpoint titers for hemagglutination activity (8HAU/50 ul) for each strain. mAbs 1E11 and 5F5 inhibited hemagglutination activity of homologous as well as heterologous viruses; mAb 4F5 demonstrated hemagglutination inhibition only against homologous virus.

Example 8: Analysis of binding activity of mAbs 13B8, 4F5, 5F5, 1E11, 1E5 and 2C7

[0334] Ascites produced and purified mAbs 13B8, 4F5, 5F5, 1E11, 1E5 and 2C7 were screened for binding activity against a panel of inactivated whole viruses, purified plant-produced HAs and baculovirus produced HAs. The results of this experiment are shown in Figure 4. All anti-H5 mAbs were shown to bind multiple H5N1 virus strains from both Clade 1 and Clade 2. None of the anti-H5 mAbs bound to influenza viruses of subtypes H3N2 or H1N1.

Example 9: Hemagglutination inhibition activity of anti-HA mAbs

[0335] Ascites produced and purified mAbs 13B8, 4F5, 5F5, 1E11, 1E5 and 2C7 were screened for hemagglutination inhibition activity according of the method of Example 7. The

results of this analysis for the anti-H5 HA mAbs, 13B8, 4F5, 5F5, 1E11, 1E5, are shown in Figure 5; the results of this analysis for the anti-H3 HA mAb, 2C7, is shown in Figure 6.

Example 10: Evaluation of anti-HA mAbs in vivo

[0336] The ability of the anti-H5 HA mAb, 1E11, to protect mice from challenge with influenza virus in vivo was analyzed according to the experimental design shown in Figure 7. Female mice were challenged on Day 0 with 30 μL of $10^{6.4}$ EID_{50}/mL of H5N1 Avian Influenza virus. On Days 0-2, each animal was dosed with either 100 μL or 200 μL of mAb 2B9 (an anti-neuraminidase mAb), mAb 1E11, combination of mAb 2B9 and mAb 1E11, or DPBS intravenously into the tail vein. On Day 0, Groups 1, 3, 5, and 7 were dosed beginning approximately 1 hour after challenge, and Groups 2, 4, 6, and 8 were dosed beginning approximately 6 hours after challenge. Mice were assessed for clinical signs, body weight, and body temperature changes throughout the study (Day 0 to Day 14). The results of this study are shown in Figure 8. Treatment of H5N1 challenged mice with monoclonal antibodies 2B9 and/or 1E11 at 1 (Figure 8A) or 6 (Figure 8B) hours after challenge provided 50%-90% protection from lethality. In general, mAb 1E11 provided a higher level of protection by 20%-30% compared to the 2B9 antibody. The timing for the administration of the mAb did not have a substantial impact on survival or body temperature. The timing for the administration of the mAb had only a modest impact on body weight for groups treated with the 2B9 antibody, with animals dosed 6 hours after challenge appearing more stable in body weight at study termination, indicating a better performance of the antibody.

Example 11: Half-life study of mAb1E11 in mice

[0337] The stability of mAb 1E11 was measured in mice according to standard methods. The half-life of the antibody was 8.4 days when administered intravenously and 13.7 days when administered intramuscularly.

Example 12: Amino acid sequences of mAbs 1E11 and 4F5

[0338] The nucleotide sequences of the light and heavy chains of mAbs 1E11 and 4F5 were determined by standard methods. Conceptual translations of each sequence are shown in Figure 9. The heavy chain of 1E11 has the acid sequence set forth in SEQ ID NO:74; the light chain of 1E11 has the acid sequence set forth in SEQ ID NO:75. The heavy chain of 4F5 has the acid sequence set forth in SEQ ID NO:76; the light chain of 4F5 has the acid sequence set forth in SEQ ID NO:77. Signal peptide/leader sequences are shown in italics.

[0339] Sequences of the 1E11 and 4F5 heavy and light chains without signal peptide/leader sequences are shown in Figure 10. The heavy chain of 1E11 without the signal peptide/leader sequence has the acid sequence set forth in SEQ ID NO:78; the light chain of 1E11 without the signal peptide/leader sequence has the acid sequence set forth in SEQ ID NO:79. The heavy chain of 4F5 without the signal peptide/leader sequence has the acid sequence set forth in SEQ ID NO:80; the light chain of 4F5 without the signal peptide/leader sequence has the acid sequence set forth in SEQ ID NO:81.

SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 60412-4580 Seq 28-MAR-12 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

CLAIMS:

1. An isolated monoclonal antibody that specifically binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody comprises the light chain variable region amino acid sequence as set forth in amino acids 1-97 of SEQ ID NO:79 and the heavy chain variable region amino acid sequence as set forth in amino acids 1-115 of SEQ ID NO:78.

2. An antibody that specifically binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody comprises the light chain amino acid sequence as set forth in SEQ ID NO:79 and the heavy chain amino acid sequence as set forth in SEQ ID NO:78.

3. The antibody of claim 1 or claim 2, wherein the antibody is an scFv, Fv, Fab', Fab, diabody, linear antibody or F(ab')2 antigen-binding fragment of an antibody.

4. The antibody of claim 1 or claim 2, wherein the antibody is a univalent fragment.

5. The antibody of claim 1 or claim 2, wherein the antibody is a human, humanized or part-human antibody or antigen-binding fragment thereof.

6. The antibody of claim 1 or claim 2, wherein the antibody is a recombinant antibody.

7. The antibody of claim 1 or claim 2, wherein the antibody is produced in a plant.

8. The antibody of any one of claims 1-7, wherein the antibody is operatively attached to a biological agent or a diagnostic agent.

9. The antibody of any one of claims 1-7, wherein the antibody is operatively attached to an agent that cleaves a substantially inactive prodrug to release a substantially active drug.

10. The antibody of claim 9, wherein the drug is an anti-influenza agent.

11. The antibody of claim 8, wherein the biological agent is an anti-viral agent.

12. The antibody of claim 11, wherein the anti-viral agent is an anti-influenza agent.

13. The antibody of claim 8, wherein the antibody is operatively attached to the biological agent as a fusion protein prepared by expressing a recombinant vector that comprises, in the same reading frame, a DNA segment encoding the antibody operatively linked to a DNA segment encoding the biological agent.

14. The antibody of claim 8, wherein the antibody is operatively attached to the biological agent via a biologically releasable bond or selectively cleavable linker.

15. The antibody of claim 8, wherein the diagnostic agent is an imaging or a detectable agent.

16. The antibody of claim 15, wherein the imaging or detectable agent is an X-ray detectable compound, a radioactive ion or a nuclear magnetic spin-resonance isotope.

17. The antibody of claim 16, wherein:

(a) the X-ray detectable compound is bismuth (III), gold (III), lanthanum (III) or lead (II);

(b) the radioactive ion is copper^{67}, gallium^{67}, gallium^{68}, indium^{111}, indium^{113}, iodine^{123}, iodine^{125}, iodine^{131}, mercury^{197}, mercury^{203}, rhenium^{186}, rhenium^{188}, rubidium^{97}, rubidium^{103}, technetium^{99m} or yttrium^{90}; or

(c) the nuclear magnetic spin-resonance isotope is cobalt (II), copper (II), chromium (III), dysprosium (III), erbium (III), gadolinium (III), holmium (III), iron (II), iron (III), manganese (II), neodymium (III), nickel (II), samarium (III), terbium (III), vanadium (II) or ytterbium (III).

18. The antibody of claim 8, wherein the antibody is operatively attached to biotin, avidin or to an enzyme that generates a colored product upon contact with a chromogenic substrate.

19. A nucleic acid comprising a nucleotide sequence encoding the antibody light chain or antibody heavy chain of the isolated monoclonal antibody of claim 1 or claim 2.

20. An expression vector comprising the nucleic acid of claim 19.

21. The expression vector of claim 20, further comprising a nucleotide sequence encoding a leader sequence.

22. A host cell comprising the expression vector of claim 20 or 21.

23. The host cell of claim 22, wherein the host cell is a plant cell.

24. The host cell of claim 23, wherein the plant cell is from a genus selected from the group consisting of *Brassica*, *Nicotiana*, *Petunia*, *Lycopersicon*, *Solanum*, *Capsium*, *Daucus*, *Apium*, *Lactuca*, *Sinapis*, and *Arabidopsis*.

25. The host cell of claim 23, wherein the plant cell is from a species selected from the group consisting of *Nicotiana benthamiana*, *Brassica carinata*, *Brassica juncea*, *Brassica napus*, *Brassica nigra*, *Brassica oleraceae*, *Brassica tournifortii*, *Sinapis alba*, and *Raphanus sativus*.

26. The host cell of claim 23, wherein the plant cell is from a plant selected from the group consisting of alfalfa, radish, mustard, mung bean, broccoli, watercress, soybean, wheat, sunflower, cabbage, clover, petunia, tomato, potato, tobacco, spinach, and lentil.

27. Use of a plant comprising the host cell of claim 23 for the production of the antibody light chain or antibody heavy chain of the isolated monoclonal antibody of claim 1 or claim 2.

28. The use of claim 27, wherein the plant is from a genus selected from the group consisting of *Brassica*, *Nicotiana*, *Petunia*, *Lycopersicon*, *Solanum*, *Capsium*, *Daucus*, *Apium*, *Lactuca*, *Sinapis*, and *Arabidopsis*.

29. A recombinant, plant-produced monoclonal antibody that specifically binds hemagglutinin, wherein the antibody has the ability to inhibit hemagglutination, and wherein the antibody comprises the light chain amino acid sequence as set forth in SEQ ID NO:79 and the heavy chain amino acid sequence as set forth in SEQ ID NO:78.

30. A pharmaceutical composition comprising the antibody of any one of claims 1-14 and 29, and a pharmaceutically acceptable carrier.

31. The pharmaceutical composition of claim 30, wherein the pharmaceutical composition is formulated for parenteral or topical administration.

32. The pharmaceutical composition of claim 30, wherein the antibody is a recombinant, plant-produced antibody.

33. The pharmaceutical composition of claim 30, wherein the pharmaceutical composition is an encapsulated or liposomal formulation.

34. The pharmaceutical composition of claim 30, wherein the pharmaceutical composition comprises as a first therapeutic agent the antibody of any one of claims 1-14 and 29 and further comprises a second therapeutic agent.

35. Use of the pharmaceutical composition of claim 30 for treating an influenza infection in a subject in need thereof, wherein the influenza infection is caused by an H5 influenza virus.

36. Use of the pharmaceutical composition of claim 30 in the manufacture of a medicament for treating an influenza infection, wherein the influenza infection is caused by an H5 influenza virus.

37. Use of the antibody of any one of claims 1-8, 15-18, and 29 for determining whether a subject is at risk for influenza virus infection, wherein the influenza infection is caused by an H5 influenza virus.

38. A method for typing an influenza virus, comprising contacting the influenza virus with the antibody of any one of claims 1-8, 15-18, and 29, and if binding of the antibody to the influenza virus is detected, typing the influenza virus as an H5 virus.

39. Use of the antibody of any one of claims 1-14 and 28 for treating influenza in a subject in need thereof, wherein the influenza infection is caused by an H5 influenza virus, and wherein the subject has been selected for treatment by a process comprising contacting a biological sample from the subject with the antibody of any one of claims 1-8, 15-18, and 29 and, if the antibody shows detectable binding to the biological sample, selecting the subject for treatment with the antibody.

40. The method of claim 37 or the use of claim 39, wherein the subject is human.

41. The use of claim 39, wherein the subject is diagnosed as having influenza.

Figure 1

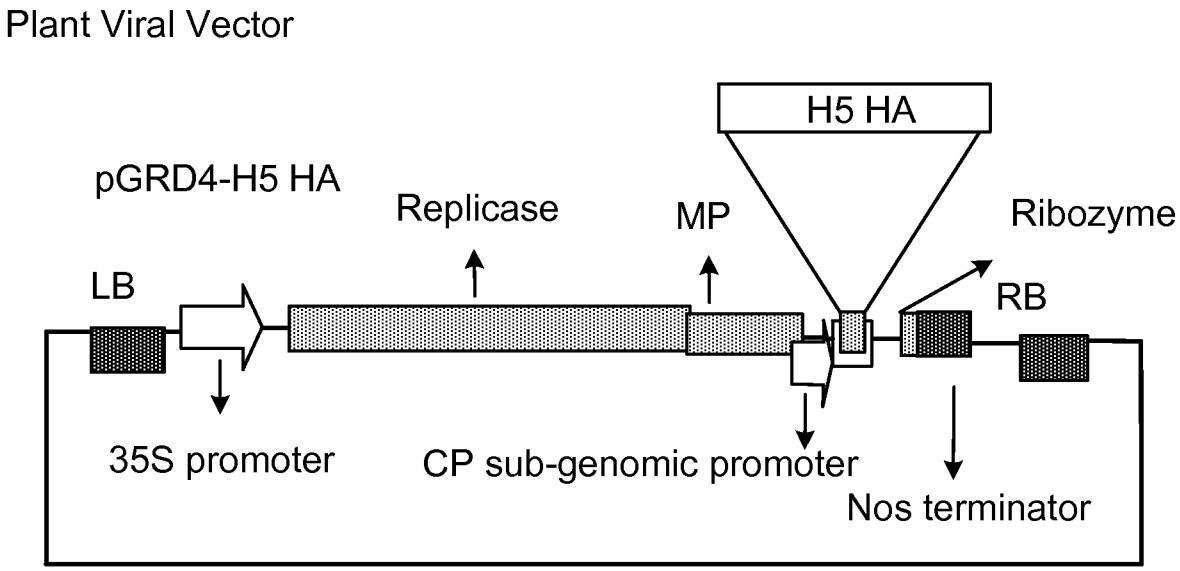
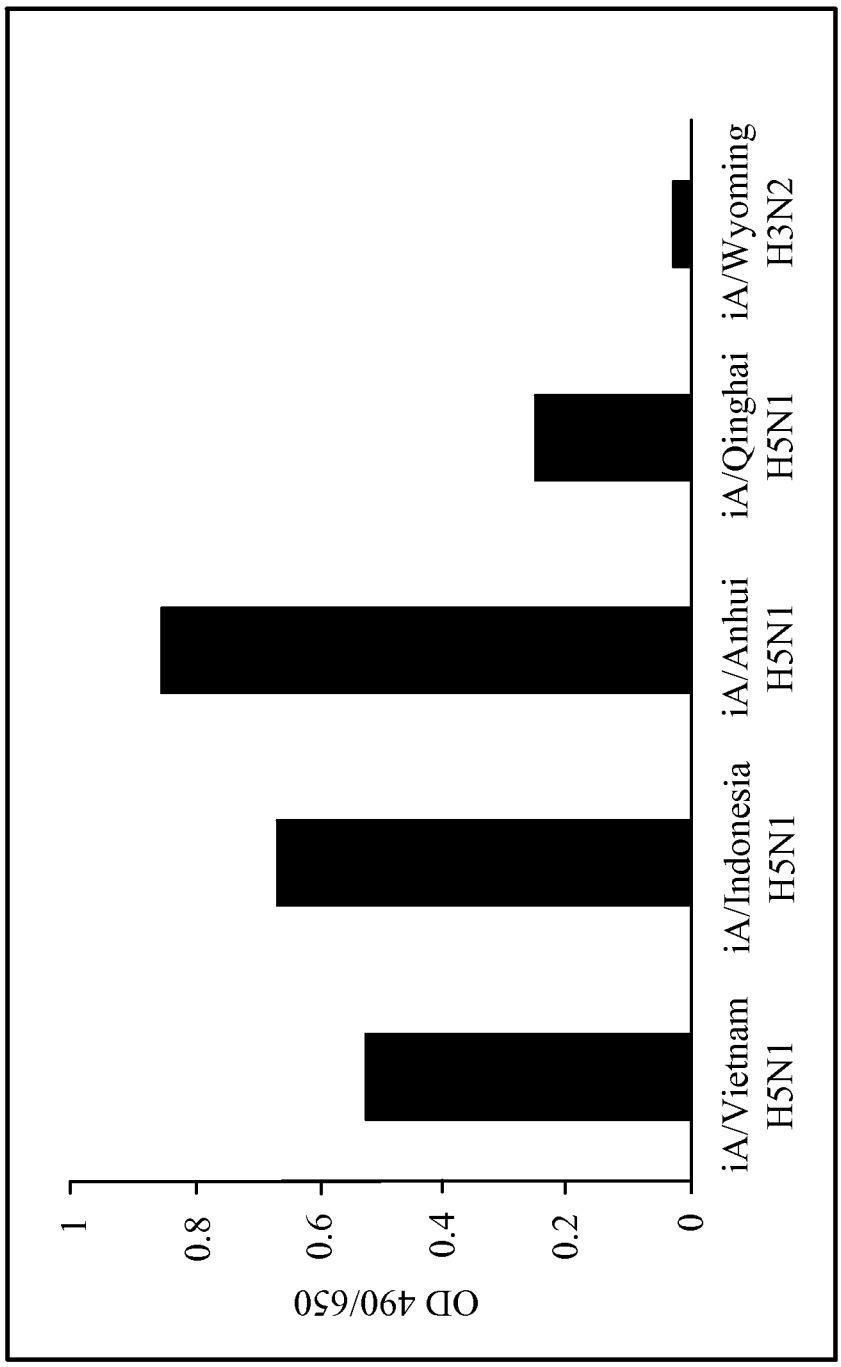

Plant Viral Vector
H5 HA
pGRD4-H5 HA
Replicase
MP
Ribozyme
LB
RB
35S promoter
CP sub-genomic promoter
Nos terminator

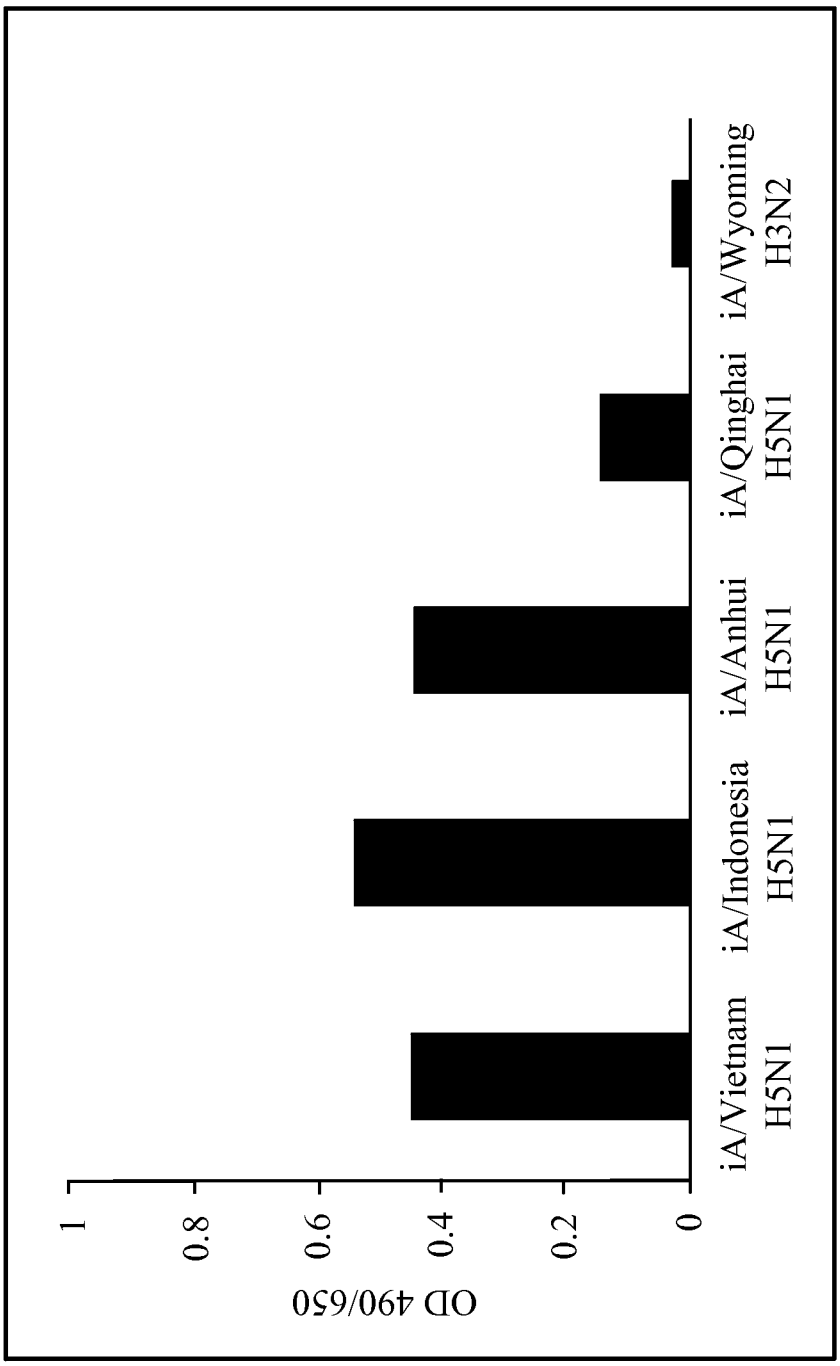
Figure 2A: Specificity of mAbs



The specificity of each monoclonal antibody to homologous and heterologous viruses was analyzed by ELISA. Plates were coated with inactivated H5N1 or H3N2 virus. Data are shown as OD 490/650 absorbance values with each monoclonal antibody concentration at 125ng/ml. These antibodies were characterized to be of subclass IgG_1, k

Figure 2B: Specificity of mAbs

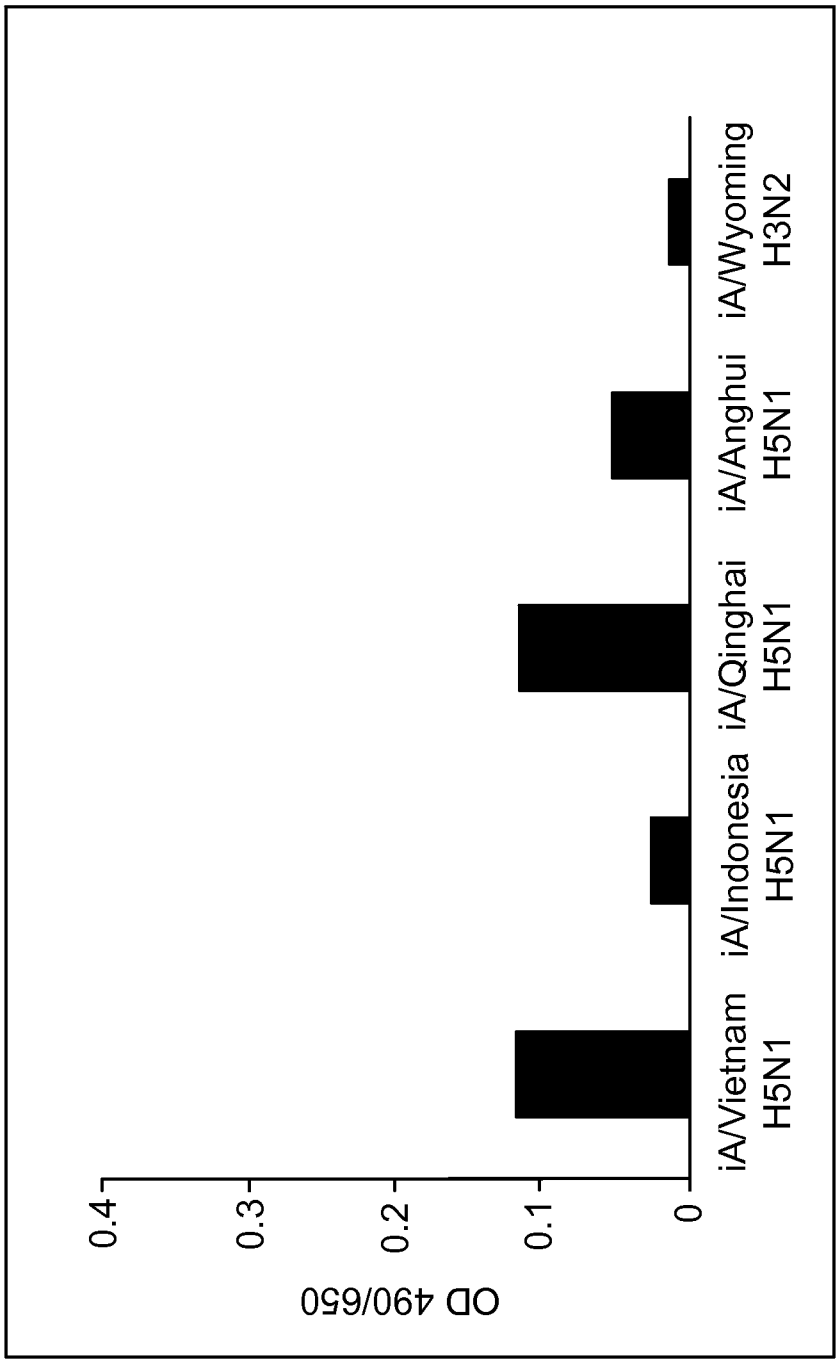
mAb 5F5 (A/Bar-headed goose/Qinghai/1A/05)



The specificity of each monoclonal antibody to homologous and heterologous viruses was analyzed by ELISA. Plates were coated with inactivated H5N1 or H3N2 virus. Data are shown as OD 490/650 absorbance values with each monoclonal antibody concentration at 125ng/ml. These antibodies were characterized to be of subclass IgG_1, k

Figure 2C: Specificity of mAbs

mAb 1E11 (A/Bar-headed goose/Qinghai/1A/05)



The specificity of each monoclonal antibody to homologous and heterologous viruses was analyzed by ELISA. Plates were coated with inactivated H5N1 or H3N2 virus. Data are shown as OD 490/650 absorbance values with each monoclonal antibody concentration at 125ng/ml. These antibodies were characterized to be of subclass IgG_1, k

Figure 3
Hemagglutination Inhibition of mAbs

Cross-clade HI titers

mAb	Antigen	A/Vietnam/ 1194/04 Clade 1	A/Indonesia/ 05/05 Clade 2.1	A/B-H Goose Qinghai/1A/05 Clade 2.2	A/turkey/ Turkey/1/05 Clade 2.2	A/Anhui/1/05 Clade 2.3
1 E 11	HAQ1	20.3 ± 4.7	81.25 ± 18.75	< 1.56	< 1.56	10.15 ± 2.35
5 F 5	HAQ1	187.5 ± 62.5	> 1000	> 1000	375 ± 125	375 ± 125
4 F 5	HAA1	> 1000	> 1000	> 1000	> 1000	23.4 ± 7.8
Sheep anti-A/Vietnam/1194/04		1280	320	640	640	640

Data are shown at the lowest antibody concentration (μg/ml) with standard deviations that inhibit hemagglutination activity (8HAU/50μl) of each strain.
For the reference serum, data are shown as endpoint titers that inhibit hemagglutination activity (8HAU/50μl) of each strain.

Figure 4

Influenza Hybridoma ELISA Data

ND=not done

pp=plant-produced

Immuni-zation	Clone	Isotype	*iA*/ NIBRG-14	*iA*/ Anhui	*iA*/ Indonesia	*iA*/ Qinghai	*iA*/ Wyoming	*iA*/ Hiroshima	*iA*/ Brisbane (H3)	*iA*/ Brisbane (H1)
HAA1	13B8	IgG2a	1.953	1.953	1.953	ND	–	–	–	–
	4F4	IgG1	7.813	11.719	3.906	ND	–	–	–	–
HAQ1	5F5	IgG1	156.25	156.25	7.813	ND	–	–	–	–
	1E11	IgG1	125	250	>1ug/ml	156	–	–	–	–
HAI	1E5	IgG1	>1ug/ml	250	98	>1ug/ml	–	–	–	–
HAWY1	2C7	IgG1	–	–	–	–	Yes	Yes	–	–

iA = inactive virus

Endpoint titers expressed in ng/ml

	Clone	Isotype	ppHAV1	ppHAA 1	ppHAI1	ppHAQ1	ppHAWY1	Baculovirus produced HAI	Baculovirus produced HAV	Baculovirus produced HAWY
HAA1	13B8	IgG2a	ND	1.953	1.953	1.953	–	19.5	19.5	–
	4F4	IgG1	ND	1.953	1.953	1.953	–	19.5	19.5	–
HAQ1	5F5	IgG1	ND	1.953	1.953	1.953	–	19.5	19.5	–
	1E11	IgG1	62.5	125	–	<156	–	Yes	Yes	–
HAI	1E5	IgG1	15.6	62.5	0.24	3.906	–	Yes	Yes	–
HAWY1	2C7	IgG1	–	–	–	–	<156	–	–	Yes

These monoclonal antibodies were produced by hybridoma cells lines generated from mice immunized with purified recombinant plant-produced hemagglutinin proteins from different strains of influenza, both pandemic as well as seasonal. Ascites produced and purified antibodies were tested for their ability to bind homologous as well as heterologous recombinant hemagglutinin proteins or inactivated influenza viruses by ELISA. A “Yes” in the charts above indicates a positive signal over background in the ELISA assay. A number indicates the endpoint titer of antibody that can bind the coating material in the assay. Unless otherwise noted the titers are expressed in ng/ml.

HI activities of anti-H5 HA mAbs

mAb	Antigen	Viruses				
		A/Anhui/1/05	A/B-H Goose Qinghai/1A/05	A/Indonesia/ 05/05	A/turkey/ Turkey/1/05	A/Vietnam /1194/04
1 E 11	HAQ1	10.15 ± 2.35	< 1.56	81.25 ± 18.75	<1.56	20.3 ± 4.7
1 E 5	HAI1	> 1000	> 1000	> 1000	> 1000	> 1000
5 F 5	HAQ1	375 ± 125	> 1000	> 1000	375 ± 125	187.5 ± 62.5
4 F 5	HAA1	23.4 ± 7.8	> 1000	> 1000	> 1000	> 1000
13 B 8	HAA1	> 1000	> 1000	> 1000	> 1000	> 1000
Sheep anti-A/Vietnam/1194/04		640	640	320	640	1280

Data are shown as the lowest antibody concentration (mg/ml) that inhibit hemagglutination activity (8 HAU/50 ml) of each strain.

For reference serum, data are shown as endpoint titers that inhibit hemagglutination activity (8HAU/50 ml) of each strain.

Figure 5

Figure 6

HI activities of anti-H3 HA mAb

mAb	Antigen	Viruses					
		A/Brisbane/ 10/07	A/California/ 07/04	A/New York/ 55/04	A/Sydney/ 5/97	A/Wisconsi n/67/05	A/Wyoming 03/03
2 C 7	HAWY1	3.2 ± 0.7	15.6 ± 0	3.2 ± 0.7	500 ± 0	3.2 ± 0.7	3.2 ± 0.7
Reference (sheep anti-Wyoming)		1280	1280	640	640	1280	1280

Data are shown as the lowest antibody concentration (μg/ml) that inhibit hemagglutination activity (8 HAU/50 μl) of each strain.

For reference serum, data are shown as endpoint titers that inhibit hemagglutination activity (8HAU/50 μl) of each strain.

Experimental Design to Evaluate the Protective Efficacy of 2B9 & 1E11

Species: Babl/c mice
Route of Ab administration: i.v.
Route of A/VN/1203/04 challenge ($10LD_{50}$): i.n.

Group	Antibodies	Dose (μg)
1	2B9	500
2	1E11	500
3	2B9 + 1E11	500 + 500
4	PBS	N/A

Figure 7

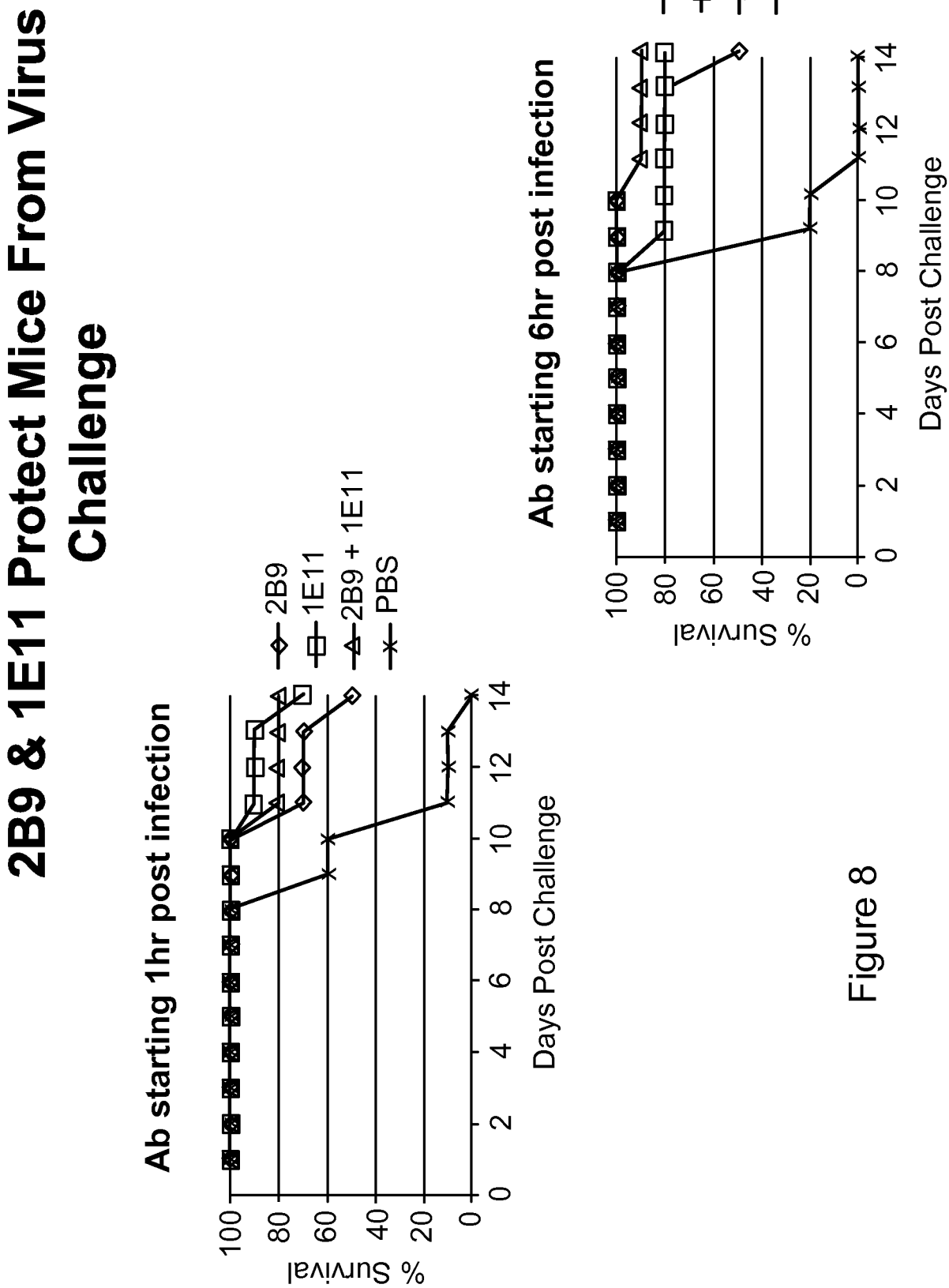

2B9 & 1E11 Protect Mice From Virus Challenge
Ab starting 1hr post infection
% Survival
Days Post Challenge
2B9
1E11
2B9 + 1E11
PBS
Ab starting 6hr post infection
% Survival
Days Post Challenge
2B9
1E11
2B9 + 1E11
PBS

Figure 8

Figure 9

1E11 heavy chain (full length sequence):

MEWSWIFLFLLSGTAGVHSEVQLQQSGPELVKPGASVKMSCKAS**GYTFTSYV**MHWVKQKPG
QGLEWIGY**INPYNDGT**RYNEKFRVKATLTSDKSSSTAYMELSSLTSEDSAVYYC**ARRGLITTPT**
LDYWGQGTTLTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSS
GVHTFPAVLQSDLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVP
EVSSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTF
RSVSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVS
LTCMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCS
VLHEGLHNHHTEKSLSHSPGK (SEQ ID NO:74)

1E11 light chain (full length sequence):

MMSSAQFLGLLLLCFQGTRCDIQMTQTSSSLSASLGDRVTISCRAS**QDIRNY**LNWYQQKPDG
TVKLLIY**YTS**RLHSGVPSRFSDSGSGTDYSLTISNLEQEDLATYFC**QQTYTLP**WTFGGGTKLEIK
RADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKD
STYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO:75)

4F5 heavy chain (full length sequence):

MKLWLNWIFLVTLLNDIQCEVKLVESGGGLVQPGGSLRLSCATS**GFTFSDYY**MSWVRQSPGK
ALEWLGF**TRSRVLGYTT**DYSASVKGRFTISRDNSQSILYLQMNSLRGEDSATYYC**ARDRPMDY**
WGQGTSVTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVH
TFPAVLQSDLYTLSSSVTVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVS
SVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRS
VSELPIMHQDWLNGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLT
CMITDFFPEDITVEWQWNGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVL
HEGLHNHHTEKSLSHSPGK (SEQ ID NO:76)

4F5 light chain (full length sequence):

MRPSIQFLGLLLFWLHGGQCDIQMTQSPSSLSASLGGNVTITCKAS**QDINKY**IAWYQHKPGKG
PRLVIH**YTS**TLQPGIPSRFSGSGSGTDYSFSISNLEPEDIATYYC**LQYDILYT**FGGGTKLEIKRAD
AAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTY
SMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO:77)

Italics, bold – signal peptide; bold – complementarity-determining regions; underlined – variable regions; other – constant region

Figure 10

1E11 heavy chain (full length sequence):

EVQLQQSGPELVKPGASVKMSCKAS**GYTFTSYV**MHWVKQKPGQGLEWIGY**INPYNDGT**RYN
EKFRVKATLTSDKSSSTAYMELSSLTSEDSAVYYC**ARRGLITTPTLDYW**GQGTTLTVSSAKTTP
PSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSV
TVPSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLT
PKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKE
FKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQW
NGQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSP
GK (SEQ ID NO:78)

1E11 light chain (full length sequence):

DIQMTQTSSSLSASLGDRVTISCRAS**QDIRNY**LNWYQQKPDGTVKLLIY**YTS**RLHSGVPSRFSD
SGSGTDYSLTISNLEQEDLATYFC**QQTYTLP**WTFGGGTKLEIKRADAAPTVSIFPPSSEQLTSG
GASVVCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHN
SYTCEATHKTSTSPIVKSFNRNEC (SEQ ID NO:79)

4F5 heavy chain (full length sequence):

EVKLVESGGGLVQPGGSLRLSCATS**GFTFSDYY**MSWVRQSPGKALEWLGF**TRSRVLGYTT**DY
SASVKGRFTISRDNSQSILYLQMNSLRGEDSATYYC**ARDRPMDY**WGQGTSVTVSSAKTTPPS
VYPLAPGSAAQTNSMVTLGCLVKGYFPEPVTVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTV
PSSTWPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEVSSVFIFPPKPKDVLTITLTPK
VTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWLNGKEFK
CRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWN
GQPAENYKNTQPIMDTDGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPG
K (SEQ ID NO:80)

4F5 light chain (full length sequence):

DIQMTQSPSSLSASLGGNVTITCKAS**QDINKY**IAWYQHKPGKGPRLVIH**YTS**TLQPGIPSRFSGS
GSGTDYSFSISNLEPEDIATYYC**LQYDILYT**FGGGTKLEIKRADAAPTVSIFPPSSEQLTSGGASV
VCFLNNFYPKDINVKWKIDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCE
ATHKTSTSPIVKSFNRNEC (SEQ ID NO:81)

Bold – complementarity-determining regions; underlined – variable regions; other – constant region