

METHODS OF OPTIMIZING NUCLEOTIDE SEQUENCES ENCODING ENGINEERED INFLUENZA PROTEINS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of, and relies on the filing date of, U.S. provisional patent application number 62/172,949, filed 9 June 2015, the entire disclosure of which is incorporated herein by reference.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 9, 2016, is named 0171.0008-PCT_SL.txt and is 351,204 bytes in size.

BACKGROUND

[0003] Influenza has a long standing history of pandemics, epidemics, resurgences and outbreaks. Vaccines have been the most effective defense against influenza. However, the effort to design and manufacture vaccines that induce strain-specific immunity year-over-year has been difficult as influenza continues to cause significant health problems across the globe. Annual influenza epidemics are thought to result in between three and five million cases of severe illness and between 250,000 and 500,000 deaths every year around the world. Furthermore, currently marketed influenza vaccines must be updated annually based on predicted strains that will be present in human populations in the impending season.

[0004] Influenza virus is a member of Orthomyxoviridae family. There are three subtypes of influenza viruses, designated influenza A, influenza B, and influenza C. The influenza virion contains a segmented negative- sense RNA genome. In the case of Influenza A viruses, the RNA genome encodes the following proteins: hemagglutinin (HA), neuraminidase (NA), matrix (M1), proton ion-channel protein (M2), nucleoprotein (NP), polymerase basic protein 1 (PBI), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), and nonstructural protein 2 (NS2). The HA, NA, M1, and M2 are membrane associated, whereas NP, PBI, PB2, PA, and NS2 are nucleocapsid associated proteins. The M1 protein is the most abundant protein in influenza particles. The HA and NA proteins are envelope glycoproteins, responsible for virus attachment and penetration of the viral particles into the cell. Specifically, HA binds the influenza virus to cells with sialic acid-containing on surface structures on their membranes.

[0005] Both HA and NA proteins are the sources of the major immunodominant epitopes for virus neutralization and protective immunity, making them important components for prophylactic influenza vaccines. The generation and recovery of influenza viruses is an important step in the evaluation of functional influenza vaccine candidates.

[0006] Reverse genetics for negative-strand RNA viruses, such as the influenza virus, has permitted genetic manipulation of viral genomes in order to generate new viruses, which can be used as live, attenuated vaccines or vectors to express heterologous proteins. Reverse genetics technology allows the generation of infectious influenza virus entirely from cloned viral cDNA (Fodor et al., 1999 J Virol, 73(11):9679-9682).

[0007] Different systems were developed based on a set of plasmids capable of inducing the expression of the eight vRNAs and at least the polymerase protein complex and the nucleoprotein (NP) required for the transcription. The polymerase protein complex and NP can also be expressed either by transfection of four additional plasmids or by the use of plasmids with bidirectional promoters that allow both vRNA and mRNA synthesis through RNA polymerase I (POL 1) and II (POL 2) (Jackson et al, 2011, J Gen Virol, 92(Pt1):1-17) respectively. The total number of plasmids transfected can vary from 16 (Neuman et al, 1999, Proc Natl Acad Sci USA, 96(16):9345-9350), or 12 (Fodor et al, 1999, J Virol, 73(11):9679-9682) to 8 (Hoffmann et al, 2002, Vaccine, 20(25-26):3165-3170), depending if the strategy is unidirectional or bidirectional, and from 3 (Neumann et al, 2005, Proc Natl Acad Sci USA, 102(46):16825-16829) to 1 (Zhang et al, 2009, J Virol, 83(18):9296-9303) if plasmid(s) encode(s) several vRNA.

[0008] Most widely used influenza vaccines comprise viruses that have been chemically or physically inactivated or live viruses that have been attenuated. Examples of such vaccines are the split influenza inactivated vaccine (IIV) or live attenuated vaccine (LAIV). Manufacturing of these vaccines typically requires the recovery and propagation of a vaccine virus in embryonated hens' eggs. However, isolates of human Influenza grow very inefficiently in eggs and isolated virus frequently need to be adapted through a process that typically involves their blind passage in eggs and their reassortment with a high-yielding laboratory virus in order to increase virus/antigen yield. Two different techniques can be used to generate reassortant Influenza virus: classical reassortment and reverse genetics. Classical reassortment of Influenza A virus involves the co-infection of eggs with the vaccine virus and a high-yielding donor virus (PR8 in most cases). The resulting reassortant progeny must undergo a process of selection in order to identify the reassortant virus with the appropriate antigenic combination

and high-yielding growth phenotype. This process of selection is cumbersome and there is no guarantee that such reassortant will be obtained. In contrast to classical reassortment, reverse genetics yield a reassortant virus with a predefined combination of genes or gene constellation, and does not require further selection. Furthermore, reverse genetics can be used in the absence of a virus isolate and it is the only technique that allows the introduction of targeted gene modifications in a vaccine virus. In fact, reverse genetics has been critical in the development of Influenza H5N1 vaccine virus in which a multi-basic cleavage site had to be removed from the HA gene.

SUMMARY

[0009] Embodiments of the present invention are based on the discovery that generation of influenza vaccine virus comprising engineered Influenza proteins, which do not naturally occur, can only be achieved through reverse genetics. While most reverse genetics applications rely on PCR or RT-PCR amplification of templates from pre-existing virus, recent advances in DNA synthesis have allowed the production of viruses in the absence of a natural viral template. Wimmer et al. (2009) *Nature Biotech.* 27 (12):1163-1172; Wimmer et al. (2011) *Annu. Rev. Microbiol.* 65:583-609. In the case of influenza virus, the use of synthetic DNA and reverse genetics technology has enabled the reconstruction of the 1918 Influenza virus (Tumpey et al. (2005) *Science* 310:77-80.) and shows promise to accelerate the production of candidate vaccine viruses in response to a flu pandemic (Dormitzer et al. (2013) *Sci Tr Med* 5 (185):1-12; Verity et al. (2011) *Influenza J.* 101-109). Furthermore, candidate vaccine viruses could incorporate rationally engineered influenza proteins designed to be better immunogens than native antigens, such as the engineered influenza proteins disclosed in PCT/US2016/035594, WO2013/122827 and US Publication Nos. 2015/0044247, 2015/0017196, 2014/0147459, 2014/0127248, and 2013/0183342 and in U.S. Provisional Application 62/345,502 or 62/344,862, all of which are incorporated herein by reference.

[0010] One important limitation to the use of reverse genetics and synthetic DNA technologies to produce influenza viruses expressing engineered proteins is the requirement for a nucleotide sequence encoding such engineered proteins. Similarly, the inability to recover or rescue infectious influenza virus expressing engineered proteins may be due, in part, to the nucleotide sequence lacking the optimal sequences for efficient viral packaging. Influenza structural proteins (e.g., HA and NA) may also generate higher viral titers depending on their specific codon usage. Increased titer can be important for maximizing the success rate of viral rescue and for improving viral yield during vaccine manufacturing.

[0011] The present invention provides, among other things, methods of generating optimized nucleotide sequences encoding an engineered influenza structural protein. Also provided are methods of using the optimized nucleotide sequences to produce infectious influenza viruses, for example, in a reverse genetics system.

[0012] In some embodiments, the method of generating an optimized nucleotide sequence encoding an engineered influenza structural protein comprises:

- a) providing an amino acid sequence of the engineered influenza structural protein;
- b) reverse-translating the amino acid sequence to generate a first nucleotide sequence;
- c) identifying a second nucleotide sequence that encodes an influenza structural protein that shares a high degree of sequence identity with the engineered influenza structural protein;
- d) at every position where the codons in the first and second nucleotide sequences code for the same amino acid, changing codons in the first nucleotide sequence to match codons from the second nucleotide sequence; and
- e) at every position where the codons in the first and second nucleotide sequences code for a different amino acid, changing codons in the first nucleotide sequence to match codons that are based on structural protein-specific influenza codon usage preferences, thereby generating the optimized nucleotide sequence.

[0013] In some embodiments, the influenza structural protein that shares a high degree of sequence identity with the engineered influenza structural protein is a wild-type influenza structural protein. In some embodiments, the influenza structural protein shares the highest degree of sequence identity with the engineered influenza structural protein (i.e., is the closest match). In some embodiments, the second nucleotide sequence encodes a wild type version of the influenza structural protein and is identified from a publicly available database comprising influenza nucleotide sequences.

[0014] In some embodiments, the engineered influenza structural protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, . SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ

ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, and SEQ ID NO: 102.

[0015] In some embodiments, the method further comprises adding the 5' and 3' non-coding sequences from a high titer rescued strain (e.g., A/PuertoRico/8/34; "PR8") to the optimized nucleotide sequence. In some embodiments, the 5' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 23 and/or the 3' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 24 or wherein the 5' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 103 and/or the 3' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 104.

[0016] In some embodiments, the method further comprises exchanging the nucleotide sequence encoding the signal peptide in the optimized nucleotide sequence with a nucleotide sequence encoding the signal peptide from a high titer rescued strain (e.g., PR8). In some embodiments, the method further comprises exchanging the nucleotide sequence encoding the transmembrane domain with a nucleotide sequence encoding the transmembrane domain from a high titer rescued strain (e.g., PR8). In some embodiments, the method further comprises exchanging the nucleotide sequence encoding the cytoplasmic domain with a nucleotide sequence encoding the cytoplasmic domain from a high titer rescued strain (e.g., PR8).

[0017] In some embodiments, the amino acid sequence of the engineered influenza structural protein encoded by the optimized nucleotide sequence is the same as the amino acid sequence encoded by the first nucleotide sequence.

[0018] In some embodiments, the optimized nucleotide sequence further comprises a nucleotide sequence encoding a signal peptide, a nucleotide sequence coding for a transmembrane domain, and/or a nucleotide sequence coding for a cytoplasmic domain.

[0019] In some embodiments, the engineered influenza structural protein is an influenza type A hemagglutinin protein. In some embodiments, the hemagglutinin protein is a subtype selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and H17.

[0020] In some embodiments, the structural protein-specific influenza codon usage preferences are set forth in Tables 1-10.

[0021] In some embodiments, reverse translating the amino acid sequence to generate a first nucleotide sequence comprises use of a codon usage table specific for influenza viruses.

[0022] Also provided are methods of expressing the optimized nucleotide sequence generated by the methods described herein, the method comprising:

inserting the optimized nucleotide sequence into an expression plasmid; and
expressing the optimized nucleotide sequence to generate the engineered influenza structural protein.

[0023] Also provided are reverse genetics method for producing an infectious influenza virus, the method comprising:

transfecting mammalian cells with one or more expression vectors, wherein the one or more expression vectors comprise an optimized nucleotide sequence encoding an engineered influenza structural protein generated by the methods described herein and b) nucleotide sequences coding for influenza proteins from one or more donor viruses;

producing the infectious influenza virus.

[0024] In some embodiments, the one or more donor viruses are selected from the group consisting of A/Puerto Rico/8/34 (H1N1) (PR8), B/Lee/40, and B/Panama/45/90.

[0025] In some embodiments, the infectious influenza virus is an infectious reassortant influenza virus comprising the genetic material of one or more donor viruses. In some embodiments, the infectious reassortant influenza virus is chimeric.

[0026] In some embodiments, the method further comprises:

harvesting the infectious influenza virus; and

infecting eggs or mammalian cells with the harvested influenza virus.

[0027] Also provided are methods of preparing an influenza vaccine composition, the method comprising:

generating a seed virus by transfecting mammalian cells with a set of expression vectors, one or more of which comprises an optimized nucleotide sequence encoding an engineered influenza structural protein generated by the methods described herein;

harvesting the seed virus; and

producing infectious influenza virus by infecting eggs or mammalian cells with the seed virus;

harvesting the infectious influenza virus after multiplication in the eggs or mammalian cells;

purifying the harvested infectious influenza virus;

optionally inactivating the purified virus; and

mixing the purified virus with a pharmaceutically acceptable carrier.

[0028] Also provided are methods of inducing an immune response to one or more influenza polypeptides in a subject, the method comprising administering the influenza vaccine composition as described herein.

[0029] Also provided are optimized nucleotide sequence encoding an engineered influenza structural protein, wherein the optimized nucleotide sequence is obtained by the methods described herein. The foregoing and other objects, features, and advantages of the invention will become more apparent from the following detailed description, which proceeds with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE DRAWING

[0030] The Drawing included herein, which is comprised of the following Figures, is for illustration purposes only not for limitation.

[0031] **Figure 1** shows a flow chart of a method of generating an optimized nucleotide sequence encoding an engineered influenza according to certain embodiments of the present invention (SEQ ID NOS 33-37, respectively, in order of appearance).

[0032] **Figure 2** shows the annotated alignment of HA protein sequences of several influenza A subtype H1N1 strains (SEQ ID NOS 38-42, respectively, in order of appearance). The full length sequences of the HA proteins of the strains exemplified in Figure 2 are provided in the sequence listing as follows: A/Texas/36/1991 (SEQ ID NO: 65); A/New_Caledonia/20/1999 (SEQ ID NO: 66); A/Solomon_Islands/3/2006 (SEQ ID NO: 67); A/Brisbane/59/2007 (SEQ ID NO: 68); and A/California/07/2009 (SEQ ID NO: 69).

Figure 3 shows the annotated alignment of HA protein sequences of several influenza A subtype H3N2 strains (SEQ ID NOS 43-47, respectively, in order of appearance). The full length sequences of the HA proteins of the strains exemplified in Figure 3 are provided in the sequence listing as follows: A/Wisconsin/67/2005 (SEQ ID NO: 70); A/Victoria/361/2011 (SEQ ID NO: 71); A/Texas/50/2012 (SEQ ID NO: 72); A/Perth/16/2009 (SEQ ID NO: 73); and A/Hong_Kong/1/1968 (SEQ ID NO: 74).

[0033] **Figure 4** shows the aligned amino acid sequences of the transmembrane region (amino acid residues 183-212) of representatives of 14 subtypes and additional subtype H3 sequences of type A influenza hemagglutinins (SEQ ID NOS 48-57 and 57-64, respectively, in order of appearance) . The usual single-letter amino acid codes are used. Dashes are introduced to maximize sequence alignment. Letters in boldface refer to residues that are conserved in 50% or more of the sequences of all different subtypes, including a few conservative replacements as described in the text. Residues are numbered using the X:31 HA2 numbering system.

DEFINITIONS

[0034] In order for the present invention to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms are set forth through the specification.

[0035] *Adjuvant*: As used herein, the term “adjuvant” refers to a substance or vehicle that non-specifically enhances the immune response to an antigen. Adjuvants can include a suspension of minerals (alum, aluminum salts, aluminum hydroxide, or phosphate) on which antigen is adsorbed; or water -in-oil emulsion in which antigen solution is emulsified in mineral oil (for example, Freund's incomplete adjuvant), sometimes with the inclusion of killed mycobacteria (Freund's complete adjuvant) to further enhance antigenicity. Immunostimulatory oligonucleotides (such as those including a CpG motif) can also be used as adjuvants (for example, see U.S. Patent Nos. 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; and 6,429,199). Adjuvants also include biological molecules, such as lipids and costimulatory molecules. Exemplary biological adjuvants include AS04 (Didierlaurent, A.M. et al, *J. Immunol.*, 2009, 183: 6186-6197), IL-2, RANTES, GM-CSF, TNF- α , IFN- γ , G-CSF, LFA-3, CD72, B7-1, B7-2, OX-40L and 41 BBL.

[0036] *Administer*: As used herein, “administering” a composition to a subject means to give, apply or bring the composition into contact with the subject. Administration can be accomplished by any of a number of routes, such as, for example, topical, oral, subcutaneous, intramuscular, intraperitoneal, intravenous, intrathecal and intradermal.

[0037] *Antibody*: As used herein, the term “antibody” refers to a polypeptide that includes canonical immunoglobulin sequence elements sufficient to confer specific binding to a particular target antigen. In some embodiments, as used herein, the term “antibody” also refers to an “antibody fragment” or “antibody fragments”, which includes a portion of an intact

antibody, such as, for example, the antigen-binding or variable region of an antibody. Examples of “antibody fragments” include Fab, Fab’, F(ab’)₂, and Fv fragments; triabodies; tetrabodies; linear antibodies; single-chain antibody molecules; and CDR-containing moieties included in multi-specific antibodies formed from antibody fragments. Those skilled in the art will appreciate that the term “antibody fragment” does not imply and is not restricted to any particular mode of generation. An antibody fragment may be produced through use of any appropriate methodology, including but not limited to cleavage of an intact antibody, chemical synthesis, recombinant production, etc. As is known in the art, intact antibodies as produced in nature are approximately 150 kD tetrameric agents comprised of two identical heavy chain polypeptides (about 50 kD each) and two identical light chain polypeptides (about 25 kD each) that associate with each other into what is commonly referred to as a “Y-shaped” structure. Each heavy chain is comprised of at least four domains (each about 110 amino acids long)—an amino-terminal variable (V_H) domain (located at the tips of the Y structure), followed by three constant domains: C_H1, C_H2, and the carboxy-terminal C_H3 (located at the base of the Y’s stem). A short region, known as the “switch”, connects the heavy chain variable and constant regions. The “hinge” connects C_H2 and C_H3 domains to the rest of the antibody. Two disulfide bonds in this hinge region connect the two heavy chain polypeptides to one another in an intact antibody. Each light chain is comprised of two domains – an amino-terminal variable (V_L) domain, followed by a carboxy-terminal constant (C_L) domain, separated from one another by another “switch”. Intact antibody tetramers are comprised of two heavy chain-light chain dimers in which the heavy and light chains are linked to one another by a single disulfide bond; two other disulfide bonds connect the heavy chain hinge regions to one another, so that the dimers are connected to one another and the tetramer is formed. Naturally-produced antibodies are also glycosylated, typically on the C_H2 domain. Each domain in a natural antibody has a structure characterized by an “immunoglobulin fold” formed from two beta sheets (e.g., 3-, 4-, or 5-stranded sheets) packed against each other in a compressed antiparallel beta barrel. Each variable domain contains three hypervariable loops known as “complement determining regions” (CDR1, CDR2, and CDR3) and four somewhat invariant “framework” regions (FR1, FR2, FR3, and FR4). When natural antibodies fold, the FR regions form the beta sheets that provide the structural framework for the domains, and the CDR loop regions from both the heavy and light chains are brought together in three-dimensional space so that they create a single hypervariable antigen binding site located at the tip of the Y structure. Amino acid sequence comparisons among antibody polypeptide chains have defined two light chain (κ and λ) classes, several heavy chain (e.g., μ, γ, α, ε, δ) classes, and certain heavy chain subclasses

($\alpha 1$, $\alpha 2$, $\gamma 1$, $\gamma 2$, $\gamma 3$, and $\gamma 4$). Antibody classes (IgA [including IgA1, IgA2], IgD, IgE, IgG [including IgG1, IgG2, IgG3, IgG4], IgM) are defined based on the class of the utilized heavy chain sequences. For purposes of the present invention, in certain embodiments, any polypeptide or complex of polypeptides that includes sufficient immunoglobulin domain sequences as found in natural antibodies can be referred to and/or used as an “antibody”, whether such polypeptide is naturally produced (e.g., generated by an organism reacting to an antigen), or produced by recombinant engineering, chemical synthesis, or other artificial system or methodology. In some embodiments, an antibody is monoclonal; in some embodiments, an antibody is polyclonal. In some embodiments, an antibody has constant region sequences that are characteristic of mouse, rabbit, primate, or human antibodies. In some embodiments, an antibody sequence elements are humanized, primatized, chimeric, etc., as is known in the art. Moreover, the term “antibody” as used herein, will be understood to encompass (unless otherwise stated or clear from context) can refer in appropriate embodiments to any of the art-known or developed constructs or formats for capturing antibody structural and functional features in alternative presentation. For example, in some embodiments, the term can refer to bi- or other multi-specific (e.g., zybodies, etc.) antibodies, Small Modular ImmunoPharmaceuticals (“SMIPs™”), single chain antibodies, camelid antibodies, and/or antibody fragments. In some embodiments, an antibody may lack a covalent modification (e.g., attachment of a glycan) that it would have if produced naturally. In some embodiments, an antibody may contain a covalent modification (e.g., attachment of a glycan, a payload [e.g., a detectable moiety, a therapeutic moiety, a catalytic moiety, etc.], or other pendant group [e.g., poly-ethylene glycol, etc.]).

[0038] Antigen: As used herein, the term “antigen”, refers to an agent that elicits an immune response; and/or (ii) an agent that is bound by a T cell receptor (e.g., when presented by an MHC molecule) or to an antibody (e.g., produced by a B cell) when exposed or administered to an organism. In some embodiments, an antigen elicits a humoral response (e.g., including production of antigen-specific antibodies) in an organism; alternatively or additionally, in some embodiments, an antigen elicits a cellular response (e.g., involving T-cells whose receptors specifically interact with the antigen) in an organism. It will be appreciated by those skilled in the art that a particular antigen may elicit an immune response in one or several members of a target organism (e.g., mice, rabbits, primates, humans), but not in all members of the target organism species. In some embodiments, an antigen elicits an immune response in at least about 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the members of a target organism species. In some embodiments, an antigen binds to an antibody and/or T cell receptor, and may or may not induce a particular physiological response in an organism. In some embodiments, for example, an antigen may bind to an antibody and/or to a T cell receptor in vitro, whether or not such an interaction occurs in vivo. In some embodiments, an antigen reacts with the products of specific humoral or cellular immunity, including those induced by heterologous immunogens. In some embodiments of the disclosed compositions and methods, influenza HA H5N1 protein is an antigen.

[0039] *Approximately*: As used herein, the term “approximately” or “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term “approximately” or “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100% of a possible value).

[0040] *Binding*: It will be understood that the term “binding”, as used herein, typically refers to a non-covalent association between or among two or more entities. “Direct” binding involves physical contact between entities or moieties; indirect binding involves physical interaction by way of physical contact with one or more intermediate entities. Binding between two or more entities can be assessed in any of a variety of contexts – including where interacting entities or moieties are studied in isolation or in the context of more complex systems (e.g., while covalently or otherwise associated with a carrier entity and/or in a biological system or cell).

[0041] *Broadly Reactive*: As used herein, “broadly reactive” means the protein sequence elicits an immune response in a subject that is sufficient to inhibit, neutralize or prevent infection of a broad range of influenza viruses (such as most or all influenza viruses within a specific subtype of, e.g., H1N1, H5N1, H3N2).

[0042] *Carrier*: As used herein, the term “carrier” refers to a diluent, adjuvant, excipient, or vehicle with which a composition is administered. In some exemplary embodiments, carriers can include sterile liquids, such as, for example, water and oils, including oils of petroleum, animal, vegetable or synthetic origin, such as, for example, peanut oil, soybean oil, mineral oil,

sesame oil and the like. In some embodiments, carriers are or include one or more solid components.

[0043] *COBRA*: As used herein, “COBRA,” refers to a Computationally Optimized Broadly Reactive Antigen, as described in WO2013/122827 and US Publication Nos. 2015/0044247, 2015/0017196, 2014/0147459, 2014/0127248, and 2013/0183342, all of which are hereby incorporated by reference in their entirety. COBRAs are engineered HA proteins that elicit a broadly reactive immune response to influenza virus. The amino acid sequence of COBRAs are designed through a series of HA protein alignments and subsequent generation of a consensus sequence based on selected influenza isolates, and these HA amino acid sequences do not occur in natural influenza strains.

[0044] *Codon-optimized*: As used herein, a “codon-optimized” nucleic acid sequence refers to a nucleic acid sequence that has been altered such that translation of the nucleic acid sequence and expression of the resulting protein is improved or optimized for a particular expression system. A “codon-optimized” nucleic acid sequence preferably encodes the same protein as a non-optimized parental sequence upon which the “codon-optimized” nucleic acid sequence is based. For example, a nucleic acid sequence may be “codon-optimized” for expression in mammalian cells (e.g., CHO cells, human cells, mouse cells etc.), bacterial cells (e.g., E.coli), insect cells, yeast cells or plant cells. A nucleic acid may also be codon-optimized to permit or enhance expression of infectious influenza virus in a reverse genetics system.

[0045] *Comparable*: The term “comparable”, as used herein, refers to two or more agents, entities, situations, sets of conditions, etc. that may not be identical to one another but that are sufficiently similar to permit comparison there between so that conclusions may reasonably be drawn based on differences or similarities observed. Those of ordinary skill in the art will understand, in context, what degree of identity is required in any given circumstance for two or more such agents, entities, situations, sets of conditions, etc. to be considered comparable.

[0046] *Determine*: Many methodologies described herein include a step of “determining”. Those of ordinary skill in the art, reading the present specification, will appreciate that such “determining” can utilize any of a variety of techniques available to those skilled in the art, including for example specific techniques explicitly referred to herein. In some embodiments, a determination involves manipulation of a physical sample. In some embodiments, a determination involves consideration and/or manipulation of data or information, for example utilizing a computer or other processing unit adapted to perform a relevant analysis. In some

embodiments, a determination involves receiving relevant information and/or materials from a source. In some embodiments, determining involves comparing one or more features of a sample or entity to a comparable reference.

[0047] *Engineered:* The term “engineered,” as used herein, describes a polypeptide whose amino acid sequence has been designed by man and/or whose existence and production require action of the hand of man. For example, an engineered HA polypeptide has an amino acid sequence that differs from the amino acid sequences of HA polypeptides found in natural influenza isolates. In some embodiments, an engineered HA polypeptide has an amino acid sequence that differs from the amino acid sequence of HA polypeptides included in the NCBI database.

[0048] *Epitope:* As used herein, the term “epitope” includes any moiety that is specifically recognized by an immunoglobulin (e.g., antibody or receptor) binding component in whole or in part. In some embodiments, an epitope is comprised of a plurality of chemical atoms or groups on an antigen. In some embodiments, such chemical atoms or groups are surface-exposed when the antigen adopts a relevant three-dimensional conformation. In some embodiments, such chemical atoms or groups are physically near to each other in space when the antigen adopts such a conformation. In some embodiments, at least some such chemical atoms or groups are physically separated from one another when the antigen adopts an alternative conformation (e.g., is linearized).

[0049] *Excipient:* As used herein, the term “excipient” refers to a non-therapeutic agent that may be included in a pharmaceutical composition, for example to provide or contribute to a desired consistency or stabilizing effect. Suitable pharmaceutical excipients include, for example, starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like.

[0050] *Expression:* The term “expression”, when used in reference to a nucleic acid herein, refers to one or more of the following events: (1) production of an RNA transcript of a DNA template (e.g., by transcription); (2) processing of an RNA transcript (e.g., by splicing, editing, 5’ cap formation, and/or 3’ end formation); (3) translation of an RNA into a polypeptide; and/or (4) post-translational modification of a polypeptide.

[0051] *Fusion protein:* As used herein, the term “fusion protein” refers to a protein encoded by a nucleic acid sequence engineered from nucleic acid sequences encoding at least a portion

of two different (e.g., heterologous) proteins. As persons of skill are no doubt aware, to create a fusion protein nucleic acid sequences are joined such that the resulting reading does not contain an internal stop codon. In some embodiments, fusion proteins as described herein include an influenza HA polypeptide or fragment thereof.

[0052] *Hemagglutinin (HA) polypeptide:* As used herein, the term “hemagglutinin polypeptide” (or “HA polypeptide”) refers to a polypeptide whose amino acid sequence includes at least one characteristic sequence of HA. A wide variety of HA sequences from influenza isolates are known in the art; indeed, the National Center for Biotechnology Information (NCBI) maintains a database (available through the world wide web at ncbi.nlm.nih.gov/genomes/FLU/) that, as of the filing of the present application included at least 9796 HA sequences. Those of ordinary skill in the art, referring to this database, can readily identify sequences that are characteristic of HA polypeptides generally, and/or of particular HA polypeptides (e.g., H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, or H17 polypeptides; or of HAs that mediate infection of particular hosts, e.g., avian, camel, canine, cat, civet, environment, equine, human, leopard, mink, mouse, seal, stone martin, swine, tiger, whale, etc.). For example, in some embodiments, an HA polypeptide includes one or more characteristic sequence elements found between about residues 97 and about 185, about 324 and about 340, about 96 and about 100, and/or about 130 and about 230 of an HA protein found in a natural isolate of an influenza virus.

[0053] *H1N1 HA polypeptide:* An “H1N1 HA polypeptide”, as that term is used herein, is an HA polypeptide whose amino acid sequence includes at least one sequence element that is characteristic of H1N1 and distinguishes H1N1 from other HA subtypes. Representative sequence elements can be determined by alignments as will be understood by those skilled in the art.

[0054] *H5N1 HA polypeptide:* An “H5N1 HA polypeptide”, as that term is used herein, is an HA polypeptide whose amino acid sequence includes at least one sequence element that is characteristic of H5N1 and distinguishes H5N1 from other HA subtypes. Representative sequence elements can be determined by alignments as will be understood by those skilled in the art.

[0055] *High titer rescued strain:* A “high titer rescued strain” refers to any influenza strain that can be produced at high titers (at least 1×10^6 pfu/ml) using reverse genetics methods.

High titer rescued strains are known in the art and include, but are not limited to A/PuertoRico/8/34 (PR8).

[0056] *Host*: The term “host” is used herein to refer to a system (e.g., a cell, organism, etc.) in which a polypeptide of interest is present. In some embodiments, a host is a system that is susceptible to infection with a particular infectious agent. In some embodiments, a host is a system that expresses a particular polypeptide of interest.

[0057] *Host cell*: As used herein, the phrase “host cell” refers to a cell into which exogenous DNA (recombinant or otherwise) has been introduced. For example, host cells may be used to produce the optimized influenza hemagglutinin polypeptides described herein by standard recombinant techniques. Persons of skill upon reading this disclosure will understand that such terms refer not only to the particular subject cell, but, to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term “*host cell*” as used herein. In some embodiments, host cells include any prokaryotic and eukaryotic cells suitable for expressing an exogenous DNA (e.g., a recombinant nucleic acid sequence). Exemplary cells include those of prokaryotes and eukaryotes (single-cell or multiple-cell), bacterial cells (e.g., strains of *E. coli*, *Bacillus* spp., *Streptomyces* spp., etc.), mycobacteria cells, fungal cells, yeast cells (e.g., *S. cerevisiae*, *S. pombe*, *P. pastoris*, *P. methanolica*, etc.), plant cells, insect cells (e.g., SF-9, SF-21, baculovirus-infected insect cells, *Trichoplusia ni*, etc.), non-human animal cells, human cells, or cell fusions such as, for example, hybridomas or quadromas. In some embodiments, the cell is a human, monkey, ape, hamster, rat, or mouse cell. In some embodiments, the cell is eukaryotic and is selected from the following cells: CHO (e.g., CHO K1, DXB-11 CHO, Veggie-CHO), COS (e.g., COS-7), retinal cell, Vero, CV1, kidney (e.g., HEK293, 293 EBNA, MSR 293, MDCK, HaK, BHK), HeLa, HepG2, WI38, MRC 5, Colo205, HB 8065, HL-60, (e.g., BHK21), Jurkat, Daudi, A431 (epidermal), CV-1, U937, 3T3, L cell, C127 cell, SP2/0, NS-0, MMT 060562, Sertoli cell, BRL 3A cell, HT1080 cell, myeloma cell, tumor cell, and a cell line derived from an aforementioned cell. In some embodiments, the cell comprises one or more viral genes, e.g., a retinal cell that expresses a viral gene (e.g., a PER.C6™ cell).

[0058] *Immune response*: As used herein, the term “immune response” refers to a response of a cell of the immune system, such as a B cell, T cell, dendritic cell, macrophage or polymorphonucleocyte, to a stimulus such as an antigen or vaccine. An immune response can include any cell of the body involved in a host defense response, including for example, an

epithelial cell that secretes an interferon or a cytokine. An immune response includes, but is not limited to, an innate and/or adaptive immune response. As used herein, a protective immune response refers to an immune response that protects a subject from infection (prevents infection or prevents the development of disease associated with infection). Methods of measuring immune responses are well known in the art and include, for example, measuring proliferation and/or activity of lymphocytes (such as B or T cells; e.g. by hemagglutination inhibition assays), secretion of cytokines or chemokines, inflammation, antibody production and the like.

[0059] *Immunogen*: As used herein, the term “immunogen” refers to a compound, composition, or substance which is capable, under appropriate conditions, of stimulating an immune response, such as the production of antibodies or a T cell response in an animal, including compositions that are injected or absorbed into an animal. As used herein, an “immunogenic composition” is an administerable composition comprising an immunogen (such as an HA polypeptide). “Immunogenic compositions” include, for example, vaccines. As used herein, “immunize” means to render a subject protected from an infectious disease, such as by vaccination.

[0060] *Infectious influenza virus*: By “infectious influenza virus” is meant an influenza virus which is able to replicate into a permissive cell. Methods for determining if a virus is infectious are well known by the one skilled in the art. For example, determining if a virus is infectious may be performed using the TCID₅₀ assay. The TCID₅₀ is a method to assess the amount of infectious virus in a sample (for instance an infected cell culture supernatant, or an infected allantoic fluid) by introducing incremental dilutions of the sample on permissive cells (such as MDCK or Vero cells) and determining the endpoint dilution that induces the infection of 50 % of the permissive cells using the Spearman-Kärber statistical method.

[0061] *In vitro*: As used herein, the term “in vitro” refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, etc., rather than within a multi-cellular organism.

[0062] *In vivo*: As used herein, the term “in vivo” refers to events that occur within a multi-cellular organism, such as a human and a non-human animal. In the context of cell-based systems, the term may be used to refer to events that occur within a living cell (as opposed to, for example, in vitro systems).

[0063] *Influenza virus proteins:* "Influenza virus proteins", as used herein, denote the PB1 , PB2, PA, HA, NP, NA, M1 , M2, NS1 and NS2/NEP proteins for type A influenza, PB1 , PB2, PA, HA, NP, NA, NB, M1 , BM2, NS1 and NS2/NEP proteins for type B influenza, or PB1 , PB2, PA, HEF, NP, M1 , M1 \ CM2, NS1 and NS2/NEP for type C influenza.

[0064] *Influenza structural protein:* As used herein, the term "influenza structural protein" refers to any protein associated with the influenza nucleocapsid, matrix and envelope, including the surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), and the matrix (M1) protein and proton ion-channel protein (M2), and functional or antigenic fragments thereof. By contrast, non-structural proteins of the influenza virus include influenza virus proteins necessary to form the ribonucleoprotein complex. By "influenza virus proteins necessary to form the ribonucleoprotein complex" is meant the proteins PA, PB1, PB2 and NP for type A, B or C influenza virus. Non-structural proteins also include NS1 and NS2.

[0065] *Influenza vaccine:* As used herein, the term "influenza vaccine" refers to an immunogenic composition capable of stimulating an immune response, administered for the prophylaxis, prevention, amelioration, or treatment of influenza virus infection. An influenza vaccine may include, for example, attenuated or killed influenza virus, subunit preparations thereof (i.e., split-inactivated vaccines), virus-like particles (VLPs) and/or antigenic polypeptides (e.g., the computationally optimized hemagglutinins described herein) or DNA derived from them, or any recombinant versions of such immunogenic materials. Influenza vaccines as described herein may optionally contain one or more adjuvants.

[0066] *Isolated:* As used herein, the term "isolated" refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) designed, produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are 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 more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. In some embodiments, as will be understood by those skilled in the art, a substance may still be considered "isolated" or even "pure", after having been combined with certain other

components such as, for example, one or more carriers or excipients (*e.g.*, buffer, solvent, water, etc.); in such embodiments, percent isolation or purity of the substance is calculated without including such carriers or excipients. To give but one example, in some embodiments, a biological polymer such as a polypeptide or polynucleotide that occurs in nature is considered to be “isolated” when, a) by virtue of its origin or source of derivation is not associated with some or all of the components that accompany it in its native state in nature; b) it is substantially free of other polypeptides or nucleic acids of the same species from the species that produces it in nature; c) is expressed by or is otherwise in association with components from a cell or other expression system that is not of the species that produces it in nature. Thus, for instance, in some embodiments, a polypeptide that is chemically synthesized or is synthesized in a cellular system different from that which produces it in nature is considered to be an “isolated” polypeptide. Alternatively or additionally, in some embodiments, a polypeptide that has been subjected to one or more purification techniques may be considered to be an “isolated” polypeptide to the extent that it has been separated from other components a) with which it is associated in nature; and/or b) with which it was associated when initially produced.

[0067] *Nucleic acid*: As used herein, the phrase “nucleic acid”, in its broadest sense, refers to any compound and/or substance that is or can be incorporated into an oligonucleotide chain. In some embodiments, a nucleic acid is a compound and/or substance that is or can be incorporated into an oligonucleotide chain via a phosphodiester linkage. As will be clear from context, in some embodiments, “nucleic acid” refers to individual nucleic acid residues (*e.g.*, nucleotides and/or nucleosides); in some embodiments, “nucleic acid” refers to an oligonucleotide chain comprising individual nucleic acid residues. In some embodiments, a “nucleic acid” is or comprises RNA; in some embodiments, a “nucleic acid” is or comprises DNA. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleic acid residues. In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleic acid analogs. In some embodiments, a nucleic acid analog differs from a nucleic acid in that it does not utilize a phosphodiester backbone. For example, in some embodiments, a nucleic acid is, comprises, or consists of one or more “peptide nucleic acids”, which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention. Alternatively or additionally, in some embodiments, a nucleic acid has one or more phosphorothioate and/or 5'-N-phosphoramidite linkages rather than phosphodiester bonds. In some embodiments, a nucleic acid is, comprises, or consists of one or more natural nucleosides (*e.g.*, adenosine,

thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine). In some embodiments, a nucleic acid is, comprises, or consists of one or more nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C-5 propynyl-cytidine, C-5 propynyl-uridine, 2-aminoadenosine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 2-aminoadenosine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, 2-thiocytidine, methylated bases, intercalated bases, and combinations thereof). In some embodiments, a nucleic acid comprises one or more modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose) as compared with those in natural nucleic acids. In some embodiments, a nucleic acid has a nucleotide sequence that encodes a functional gene product such as an RNA or protein. In some embodiments, a nucleic acid includes one or more introns. In some embodiments, nucleic acids are prepared by one or more of isolation from a natural source, enzymatic synthesis by polymerization based on a complementary template (*in vivo* or *in vitro*), reproduction in a recombinant cell or system, and chemical synthesis. In some embodiments, a nucleic acid is at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 225, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500, 600, 700, 800, 900, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000 or more residues long. In some embodiments, a nucleic acid is single stranded; in some embodiments, a nucleic acid is double stranded. In some embodiments a nucleic acid has a nucleotide sequence comprising at least one element that encodes, or is the complement of a sequence that encodes, a polypeptide. In some embodiments, a nucleic acid has enzymatic activity.

[0068] Operably linked: As used herein, the phrase “operably linked” refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. "Operably linked" sequences include both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest. The term "expression control sequence" as used herein refers to polynucleotide sequences which are necessary to effect the expression and processing of coding sequences to which they are ligated. Expression control sequences include appropriate transcription initiation, termination, promoter and enhancer sequences; efficient

RNA processing signals such as splicing and polyadenylation signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance protein secretion. The nature of such control sequences differs depending upon the host organism. For example, in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription termination sequence, while in eukaryotes, typically, such control sequences include promoters and transcription termination sequence. The term "*control sequences*" is intended to include components whose presence is essential for expression and processing, and can also include additional components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

[0069] *Outbreak*: As used herein, an influenza virus “outbreak” refers to a collection of virus isolates from within a single country in a given year.

[0070] *Pandemic strain*: A “pandemic” influenza strain is one that has caused or has capacity to cause pandemic infection of human populations. In some embodiments, a pandemic strain has caused pandemic infection. In some embodiments, such pandemic infection involves epidemic infection across multiple territories; in some embodiments, pandemic infection involves infection across territories that are separated from one another (e.g., by mountains, bodies of water, as part of distinct continents, etc.) such that infections ordinarily do not pass between them.

[0071] *Permissive cells*: By “permissive cells” is meant cells that allow influenza virus to both penetrate into said cells and to achieve its full replication cycle until the production of new infectious virus. Highly permissive cells are cells where influenza viruses actively replicate and produce high amounts of infectious virus.

[0072] *Pharmaceutically acceptable vehicles*: As used herein, the term “pharmaceutically acceptable carrier” means any solvent, dispersing medium, charge, etc., commonly used on the formulation of pharmaceuticals and vaccines to enhance stability, sterility and deliverability of the active agent, which does not produce any secondary reaction, for example an allergic reaction, in humans. The excipient is selected on the basis of the pharmaceutical form chosen, the method and the route of administration. Appropriate excipients, and requirements in relation to pharmaceutical formulation, are described in Remington's Pharmaceutical Sciences (19th Edition, A.R. Gennaro, Ed., Mack Publishing Co., Easton, PA (1995)), which represents

a reference work in the field. Examples of pharmaceutically acceptable excipients are water, phosphate-buffered saline solutions, 0.3% glycine solution.

[0073] *Polypeptide:* A “polypeptide”, generally speaking, is a string of at least two amino acids attached to one another by a peptide bond. In some embodiments, a polypeptide may include at least 3-5 amino acids, each of which is attached to others by way of at least one peptide bond. Those of ordinary skill in the art will appreciate that polypeptides sometimes include “non-natural” amino acids or other entities that nonetheless are capable of integrating into a polypeptide chain, optionally. In some embodiments, the term “polypeptide” is used to refer to specific functional classes of polypeptides, such as, HA polypeptides, etc. In some embodiments, a useful polypeptide may comprise or consist of a fragment of a parent polypeptide (e.g., an epitope). In some embodiments, a useful polypeptide as may comprise or consist of multiple (e.g., two, three, four, etc.) fragments (e.g., epitopes), each of which is found in the same parent polypeptide in a different spatial arrangement relative to one another than is found in the polypeptide of interest (e.g., fragments that are directly linked in the parent may be spatially separated in the polypeptide of interest or vice versa, and/or fragments may be present in a different order in the polypeptide of interest than in the parent), so that the polypeptide of interest is a derivative of its parent polypeptide. Alternatively, in some embodiments, a useful polypeptide may comprise or consist of multiple (e.g., two, three, four, etc.) fragments (e.g., epitopes), each of which is found in different parent polypeptides than the polypeptide of interest (e.g., fragments that originate in different parent polypeptides, and/or fragments may be present in a different order in the polypeptide of interest than in the parent polypeptides), so that the polypeptide of interest is a derivative of its parent polypeptides.

[0074] *Prevention:* The term “prevention”, as used herein, refers to prophylaxis, avoidance of disease manifestation, a delay of onset, and/or reduction in frequency and/or severity of one or more symptoms of a particular disease, disorder or condition (e.g., infection for example with influenza virus). In some embodiments, prevention is assessed on a population basis such that an agent is considered to “prevent” a particular disease, disorder or condition if a statistically significant decrease in the development, frequency, and/or intensity of one or more symptoms of the disease, disorder or condition is observed in a population susceptible to the disease, disorder, or condition.

[0075] *Pure:* As used herein, an agent or entity is “pure” if it is substantially free of other components. For example, a preparation that contains more than about 90% of a particular agent or entity is typically considered to be a pure preparation. In some embodiments, an agent

or entity is at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% pure.

[0076] *Reassortant virus*: The term “reassortant virus” denotes a virus which contains genetic material that results from the combination of genetic material of at least two donor viruses. As used herein, the term may describe any influenza virus comprising parts from more than one parental strain, regardless of whether the virus is made by classic reassortant recombination or reverse genetics. When the reassortant virus is used for preparing a flu vaccine, its genetic material usually contains at least the HA and NA genes from a seasonal or pandemic virus (or an engineered version thereof) whereas the other genes (backbone genes) are from one or several other donor viruses which have been selected for their ability to grow easily on the substrate of production used for manufacturing the flu vaccine (such as the allantoic cavity of embryonated hen's eggs or a permissive cell line) and/or to be less or non-pathogenic to humans. Examples of donor viruses that contribute as “provider” of backbone genes include A/Puerto Rico/8/34 (H1N1) (A/PR/8/34), B/Lee/40 and/or B/Panama/45/90 viruses.

[0077] *Receptor-Binding Site (RBS)*: As used herein, the term “receptor-binding site” or “RBS” comprises contiguous or non-contiguous amino acid residues of the head region of an influenza HA polypeptide, which include amino acids involved in direct binding of sialic acid on the target cell receptor proteins. Amino acid residues that make up a “receptor-binding site” or “RBS” of an influenza HA polypeptide may be described from crystal structures of HA polypeptides complexed with sialic acid analogs and identifying amino acid residues within a certain proximity to the analog or may be described in reference to an HA polypeptide sequence from a particular viral strain (e.g., A/New Caledonia/20/99 or A/California/07/2009). Thus, in some embodiments, the “receptor-binding site” or “RBS” of an engineered HA polypeptide as described herein may be determined using a reference HA polypeptide sequence. In some embodiments, the “receptor-binding site” or “RBS” of an engineered HA polypeptide as described herein may be determined using the crystal structures of HA polypeptide sequence in complex with human and avian receptor analogs (ex. LSTa, LSTc). An exemplary reference crystal structure of HA polypeptide sequence in complex with LSTc includes A/Puerto Rico/8/1934 (H1N1) pdb|1RVZ.

[0078] *Recombinant*: As used herein, the term “recombinant” is intended to refer to polypeptides (e.g., HA polypeptides as described herein) that are designed, engineered, prepared, expressed, created or isolated by recombinant means, such as polypeptides expressed using a recombinant expression vector transfected into a host cell, polypeptides isolated from

a recombinant, combinatorial polypeptide library or polypeptides prepared, expressed, created or isolated by any other means that involves splicing selected sequence elements to one another. In some embodiments, one or more of such selected sequence elements is found in nature. In some embodiments, one or more of such selected sequence elements is designed *in silico*. In some embodiments, one or more such selected sequence elements results from mutagenesis (e.g., *in vivo* or *in vitro*) of a known sequence element, e.g., from a natural or synthetic source. In some embodiments, one or more such selected sequence elements results from the combination of multiple (e.g., two or more) known sequence elements that are not naturally present in the same polypeptide (e.g., two epitopes from two separate H5 HA polypeptides).

[0079] Reference: The term “reference” is often used herein to describe a standard or control agent, individual, population, sample, sequence or value against which an agent, individual, population, sample, sequence or value of interest is compared. In some embodiments, a reference agent, individual, population, sample, sequence or value is tested and/or determined substantially simultaneously with the testing or determination of the agent, individual, population, sample, sequence or value of interest. In some embodiments, a reference agent, individual, population, sample, sequence or value is a historical reference, optionally embodied in a tangible medium. Typically, as would be understood by those skilled in the art, a reference agent, individual, population, sample, sequence or value is determined or characterized under conditions comparable to those utilized to determine or characterize the agent, individual, population, sample, sequence or value of interest.

[0080] Reverse genetics: The term “reverse genetics” denotes molecular methods to produce infectious, reassortant viruses, or attenuated viruses from their complementary DNAs (cDNAs). These methods are very advantageous for producing reassortant influenza viruses by reassortment of vRNAs between different influenza viruses. The reverse genetics methods are well-known by the one skilled in the art (see, e.g., Neumann, G. and Kawaoka, Y., *Virology*, 2001, 287, 243-250).

[0081] Sequence identity: The similarity between amino acid or nucleic acid sequences is expressed in terms of the similarity between the sequences, otherwise referred to as sequence identity. Sequence identity is frequently measured in terms of percentage identity (or similarity or homology); the higher the percentage, the more similar the two sequences are. Homologs or variants of a given gene or protein will possess a relatively high degree of sequence identity when aligned using standard methods. Methods of alignment of sequences for comparison are well known in the art. Various programs and alignment algorithms are described in: Smith and

Waterman, Adv. Appl. Math. 2:482, 1981; Needleman and Wunsch, J. Mol. Biol. 48:443, 1970; Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988; Higgins and Sharp, Gene 73:237-244, 1988; Higgins and Sharp, CABIOS 5:151-153, 1989; Corpet et al., Nucleic Acids Research 16:10881-10890, 1988; and Pearson and Lipman, Proc. Natl. Acad. Sci. U.S.A. 85:2444, 1988. Altschul et al., Nature Genet. 6:119-129, 1994. The NCBI Basic Local Alignment Search Tool (BLAST®) (Altschul et al., J. Mol. Biol. 215:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, Md.) and on the Internet, for use in connection with the sequence analysis programs blastp, blastn, blastx, tblastn and tblastx.

[0082] *Subject*: As used herein, the term “subject” means any mammal, including mice, ferrets and humans. In certain embodiments of the present invention the subject is an adult, an adolescent or an infant. In some embodiments, terms “individual” or “patient” are used and are intended to be interchangeable with “subject”. Also contemplated by the present invention are the co-administration of the optimized H5N1 influenza HA proteins and/or performance of the methods to/or birds, including chickens and ducks.

[0083] *Substantially*: As used herein, the term “substantially” refers to the qualitative condition of exhibiting total or near-total extent or degree of a characteristic or property of interest. One of ordinary skill in the biological arts will understand that biological and chemical phenomena rarely, if ever, go to completion and/or proceed to completeness or achieve or avoid an absolute result. The term “substantially” is therefore used herein to capture the potential lack of completeness inherent in many biological and chemical phenomena.

[0084] *Transformation*: As used herein, refers to any process by which exogenous DNA is introduced into a host cell. Transformation may occur under natural or artificial conditions using various methods well known in the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. In some embodiments, a particular transformation methodology is selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, mating, lipofection. In some embodiments, a “transformed” cell is stably transformed in that the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. In some embodiments, a transformed cell transiently expresses introduced nucleic acid for limited periods of time.

[0085] *Vaccination*: As used herein, the term “vaccination” refers to the administration of a composition (specifically co-administration of two or more of the three computationally optimized H5N1 HA polypeptides described herein) intended to generate an immune response, for example to a disease-causing agent such as influenza. Vaccination can be administered before, during, and/or after exposure to a disease-causing agent, and/or to the development of one or more symptoms, and in some embodiments, before, during, and/or shortly after exposure to the agent. Vaccines may elicit both prophylactic (preventative) and therapeutic responses. Methods of administration vary according to the vaccine, but may include inoculation, ingestion, inhalation or other forms of administration. Inoculations can be delivered by any of a number of routes, including parenteral, such as intravenous, subcutaneous or intramuscular. Vaccines may be administered with an adjuvant to boost the immune response. In some embodiments, vaccination includes multiple administrations, appropriately spaced in time, of a vaccinating composition.

[0086] *Vector*: As used herein, the term “vector” refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid”, which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as “expression vectors.”

[0087] *Virus-like particle (VLP)*: As used herein, the phrase “virus-like particle” or “VLP” refers to particles that resemble a virus yet lack any viral genetic material and, therefore, are not infectious. A “virus-like particle” or “VLP” may be produced by heterologous expression in a variety of cell culture systems including mammalian cell lines, insect cell lines, yeast, and plant cells. In addition, VLPs can be purified by methods known in the art. In some embodiments, influenza VLPs as described herein comprise hemagglutinin (HA) polypeptides and neuraminidase (NA) polypeptides. In some embodiments, influenza VLPs as described herein comprises HA polypeptides, NA polypeptides and/or viral structural polypeptides (e.g., an influenza structural protein such as influenza M1). In some certain embodiments, influenza

VLPs as described herein comprises HA polypeptides, NA polypeptides and/or M1 polypeptides. In some embodiments, influenza VLPs as described herein comprises HA polypeptides, NA polypeptides and/or HIV gag polypeptides. As persons of skill are aware, other viral structural proteins may be used as alternatives to those exemplified herein. Influenza VLPs can be produced by transfection of host cells (e.g., mammalian cells) with plasmids encoding HA and NA proteins, and optionally HIV gag proteins. After incubation of the transfected cells for an appropriate time to allow for protein expression (such as for approximately 72 hours), VLPs can be isolated from cell culture supernatants. In some embodiments, influenza VLPs as described herein are produced by transient transfection in mammalian cells (e.g., human cells). In some embodiments, influenza VLPs are analyzed by the use of one or more assays. To give but a few examples, influenza VLPs may be analyzed for hemagglutinin activity, dynamic light scattering and hemagglutinin content quantitation by protein staining. Other assays will be readily apparent to persons of skill upon reviewing the present disclosure.

[0088] *vRNA*: By “vRNA” is meant the negative-sense viral RNA of the influenza virus which is encapsulated into the ribonucleoprotein complex. When the influenza virus is of type A or B, said vRNAs are PB2, PB1, PA, HA, NP, NA, M and NS vRNAs. When the influenza virus is of type C, said vRNAs are PB1, PB2, PA, HEF, NP, M and NS vRNAs.

[0089] *cRNA*: By “cRNA” is meant the positive-sense RNA intermediate which is complementary to the vRNA. Once in the nucleus, the incoming negative-sense viral RNA (vRNA) is transcribed into messenger RNA (mRNA) by a primer-dependent mechanism. These mRNA products are incomplete copies of the vRNA template and are capped and polyadenylated, unlike vRNA. Replication occurs via a two-step process. A full-length, positive-sense copy of the vRNA is first made that is referred to as complementary RNA (cRNA) and is in turn used as a template to produce more vRNA.

[0090] *Wild type*: As is understood in the art, the phrase “wild type” generally refers to a normal form of a protein or nucleic acid, as is found in nature. For example, wild type HA polypeptides are found in natural isolates of influenza virus. A variety of different wild type HA sequences can be found in the NCBI influenza virus sequence database, available through the world wide web at ncbi.nlm.nih.gov/genomes/FLU/FLU.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0091] The present invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting unless indicated, since the scope of the present invention will be limited only by the appended claims.

[0092] Unless stated otherwise, all technical and scientific terms and phrases used herein have the same meaning as commonly understood by one of ordinary skill in the art. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference.

[0093] Standard techniques may be used for recombinant DNA, oligonucleotide synthesis, and tissue culture and transformation (e.g., electroporation, lipofection). Enzymatic reactions and purification techniques may be performed according to manufacturer's specifications or as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al. *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)), which is incorporated herein by reference for any purpose.

Generation of Optimized Nucleotide Sequences Encoding Engineered Influenza Proteins

[0094] Recent advances have allowed for the production of rationally engineered influenza proteins designed to be better immunogens than native influenza proteins. Starting with an engineered influenza protein, it is possible to reverse translate the amino acid sequence of the engineered protein to generate a nucleotide sequence that encodes the engineered protein. The nucleotide sequence can be used in a reverse genetics system to facilitate the rescue of infectious influenza viruses containing modified versions of the influenza structural proteins (e.g., hemagglutinin or neuraminidase). However, it has been found that little to no infectious influenza virus can be rescued when using certain engineered influenza proteins in a reverse genetics system. Without being bound by a particular theory, this phenomenon may be due, in part, to the nucleotide sequence encoding the engineered influenza protein lacking the optimal sequences for efficient viral packaging and/or efficient gene expression.

[0095] Disclosed herein are methods to generate an optimized nucleotide sequence encoding an engineered influenza structural protein. Optimizing the nucleotide sequence encoding the engineered influenza protein improves the likelihood of rescuing or recovering infectious influenza virus. It can also optimize virus growth and protein yield. The nucleotide sequence can be optimized through, among other things, the modification of the sequence by i) using an influenza-specific codon usage table (derived specifically for influenza structural proteins, such as hemagglutinin and neuraminidase); and/or ii) using other influenza sequences (e.g., from wild type or previously rescued strains) as templates for reverse translations.

[0096] Figure 1 provides a flow chart for certain embodiments of these methods. In these methods, the amino acid of the engineered structural protein is reverse translated into a nucleotide sequence, as shown in Step 1 of Figure 1. The sequence may be reverse translated using a standard codon usage table or a codon usage table that is specific for influenza viruses. These codon usage tables are known in the art or can be prepared by comparing influenza sequences.

[0097] As shown Step 2 of Figure 1, the first nucleotide sequence or a translation of the first nucleotide sequence is used to identify the second nucleotide sequence that encodes a corresponding influenza structural protein from a wild type virus or a previously rescued virus. That is, an initial round of comparisons to find the second sequence is performed using the first nucleotide sequence or a translated amino acid sequence thereof (e.g., against a translated nucleotide database). The nucleotide sequence of the match is then used in the downstream steps. For example, the first nucleotide sequence or a translation of the first nucleotide sequence can be used to search a database that includes influenza protein sequences or nucleotide sequences and to identify nucleotide sequences sharing a high degree of sequence identity (e.g., to identify the closest matching corresponding structural protein in a wild-type strain). The sequence similarity searching can be done using search tools, such as the NCBI Basic Local Alignment Search Tool (BLAST®) (Altschul et al., J. Mol. Biol. 215:403-410, 1990), which is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, Md.) and FASTA, which is available from several sources, including the EMBL-EBI website.

[0098] The first and second nucleotide sequences and/or translations thereof share a high degree of sequence identity. In certain embodiments, the second nucleotide sequence is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the first nucleotide sequence. In certain embodiments, the amino acid sequence encoded by the second nucleotide

sequence is at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence encoded by the first nucleotide sequence. In one embodiment, the second nucleotide sequence and/or a translation thereof share the highest degree of sequence identity with the first nucleotide sequence and/or a translation thereof from among the nucleic acids or proteins in the database (e.g., the translation of the second nucleotide sequence is the closest match to the translation of the first nucleotide sequence in terms of sequence identity). In certain embodiments, the second nucleotide sequence and a translation thereof are a wild type version of the influenza structural protein. In other embodiments, the second nucleotide sequence and a translation thereof are versions of the influenza structural protein from an influenza virus that is capable of being rescued in a reverse genetics system.

[0099] Once the second nucleotide sequence is identified, the codons are compared. As shown Step 3a of Figure 1, at every position where the codons in the first and second nucleotide sequences code for the same amino acid, the codons in the first nucleotide sequence are changed to match codons from the second nucleotide sequence. As shown in Step 3b, at every position where the codons in the first and second nucleotide sequences code for a different amino acid, the codons in the first nucleotide sequence are changed to match codons that are based on influenza protein-specific influenza codon usage preferences to generate an optimized nucleotide sequence.

[0100] In certain embodiments, the method of generating an optimized nucleotide sequence encoding an engineered influenza structural protein comprises:

- a) providing an amino acid sequence of the engineered influenza structural protein;
- b) reverse-translating the amino acid sequence to generate a first nucleotide sequence;
- c) identifying a second nucleotide sequence that encodes a version of the influenza structural protein that shares a high degree of identity with the first nucleotide sequence (e.g., a sequence from a wild type influenza virus or an influenza virus that is capable of being rescued in a reverse genetics system);
- d) at every position where the codons in the first and second nucleotide sequences code for the same amino acid, changing codons in the first nucleotide sequence to match codons from the second nucleotide sequence; and
- e) at every position where the codons in the first and second nucleotide sequences code for a different amino acid, changing codons in the first nucleotide sequence to match codons

that are based on structural protein-specific influenza codon usage preferences, thereby generating the optimized nucleotide sequence.

[0101] In general, the amino acid sequence of the engineered influenza structural protein encoded by the optimized nucleotide sequence is the same as the amino acid sequence encoded by the first, non-optimized, nucleotide sequence. However, it is within the skill of the art to introduce minor changes in the amino acid sequence of the engineered influenza structural protein encoded by the optimized nucleotide sequence relative to the amino acid sequence encoded by the first nucleotide sequence, while retaining the ability to produce an infectious influenza virus in a reverse genetics system. Thus, in certain embodiments, the amino acid sequence of the engineered influenza structural protein encoded by the optimized nucleotide sequence has no more than 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acid differences relative to the amino acid sequence encoded by the first nucleotide sequence.

[0102] Protein-specific influenza codon usage preferences can be generated by comparing influenza protein sequences. The codon usage preferences can be determined for a specific influenza structural protein (e.g., HA or NA). By way of example, exemplary protein-specific influenza codon usage preferences that have been generated by comparing influenza HA protein and nucleotide sequences are set forth below in Tables 1-5 for 1) influenza B (human), 2) influenza A H1N1 (human), 3) influenza A H1N1 (multi), 4) influenza A H3N2 (human), and 5) influenza A H3N2 (multi), where “multi” indicates that influenza sequences from multiple animal sources (e.g., human, swine, and avian) were analyzed.

[0103] Table 1: HA Influenza B (human) Codon Usage Preference

Coding GC 43.56%

1st letter GC 49.80%

2nd letter GC 43.52%

3rd letter GC 37.36%

Codon AA Fraction Frequency Number

GCA A 0.462 31.699 95258

GCC A 0.157 10.812 32492

GCG A 0.059 4.041 12143

GCT A 0.322 22.132 66508

TGC C 0.789 20.566 61803

TGT C 0.211 5.513 16566

GAC D 0.402 19.190 57667

GAT D 0.598 28.551 85798

GAA E 0.797 44.055 132391

GAG E 0.203 11.216 33705

TTC F 0.547 14.288 42938

TTT	F	0.453	11.849	35606
GGA	G	0.526	51.266	154060
GGC	G	0.100	9.725	29226
GGG	G	0.198	19.282	57943
GGT	G	0.176	17.108	51411
CAC	H	0.390	11.353	34118
CAT	H	0.610	17.735	53295
ATA	I	0.553	33.601	100975
ATC	I	0.116	7.081	21278
ATT	I	0.331	20.105	60417
AAA	K	0.648	46.166	138734
AAG	K	0.352	25.100	75429
CTA	L	0.155	14.542	43699
CTC	L	0.231	21.688	65174
CTG	L	0.151	14.179	42610
CTT	L	0.152	14.215	42717
TTA	L	0.141	13.257	39839
TTG	L	0.169	15.812	47517
ATG	M	1.000	14.684	44126
AAC	N	0.520	30.638	92069
AAT	N	0.480	28.301	85048
CCA	P	0.387	19.703	59210
CCC	P	0.203	10.335	31057
CCG	P	0.013	0.678	2037
CCT	P	0.396	20.147	60544
CAA	Q	0.798	23.125	69494
CAG	Q	0.202	5.850	17579
AGA	R	0.642	21.921	65875
AGG	R	0.281	9.596	28837
CGA	R	0.073	2.478	7448
CGC	R	0.000	0.001	3
CGG	R	0.001	0.026	79
CGT	R	0.004	0.123	371
AGC	S	0.145	9.357	28118
AGT	S	0.144	9.287	27907
TCA	S	0.295	18.986	57056
TCC	S	0.081	5.186	15585
TCG	S	0.035	2.268	6815
TCT	S	0.300	19.325	58073
ACA	T	0.498	41.030	123299
ACC	T	0.295	24.274	72945
ACG	T	0.026	2.148	6456
ACT	T	0.181	14.954	44939
GTA	V	0.231	12.203	36671
GTC	V	0.212	11.192	33633
GTG	V	0.295	15.549	46725
GTT	V	0.262	13.810	41500
TGG	W	1.000	11.185	33613
TAC	Y	0.641	16.341	49106
TAT	Y	0.359	9.170	27557

TAA	*	0.600	0.001	3
TAG	*	0.200	0.000	1
TGA	*	0.200	0.000	1

[0104] Table 2: HA Influenza A H1N1 (human) Codon Usage Preference

Coding GC 40.67%

1st letter GC 44.58%

2nd letter GC 38.14%

3rd letter GC 39.28%

Codon	AA	Fraction	Frequency	Number
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GCA	A	0.467	24.169	226427
GCC	A	0.297	15.363	143929
GCG	A	0.043	2.213	20733
GCT	A	0.194	10.032	93982
TGC	C	0.415	9.720	91067
TGT	C	0.585	13.721	128543
GAC	D	0.500	24.453	229087
GAT	D	0.500	24.407	228658
GAA	E	0.689	47.154	441772
GAG	E	0.311	21.288	199442
TTC	F	0.598	19.970	187088
TTT	F	0.402	13.442	125937
GGA	G	0.356	26.630	249488
GGC	G	0.115	8.604	80604
GGG	G	0.307	22.980	215289
GGT	G	0.223	16.664	156119
CAC	H	0.471	13.772	129023
CAT	H	0.529	15.471	144944
ATA	I	0.344	20.137	188659
ATC	I	0.141	8.251	77298
ATT	I	0.514	30.077	281779
AAA	K	0.652	52.096	488070
AAG	K	0.348	27.746	259946
CTA	L	0.275	20.519	192231
CTC	L	0.121	9.023	84533
CTG	L	0.196	14.616	136931
CTT	L	0.035	2.609	24441
TTA	L	0.152	11.315	106003
TTG	L	0.221	16.520	154767
ATG	M	1.000	10.255	96071
AAC	N	0.373	29.309	274589
AAT	N	0.627	49.326	462121
CCA	P	0.489	18.348	171892
CCC	P	0.209	7.858	73618
CCG	P	0.237	8.883	83222
CCT	P	0.065	2.426	22732
CAA	Q	0.477	13.241	124048
CAG	Q	0.523	14.528	136105

AGA	R	0.740	24.677	231194
AGG	R	0.257	8.561	80204
CGA	R	0.001	0.040	372
CGC	R	0.000	0.007	70
CGG	R	0.001	0.019	176
CGT	R	0.002	0.055	516
AGC	S	0.210	16.154	151339
AGT	S	0.128	9.836	92146
TCA	S	0.375	28.788	269704
TCC	S	0.088	6.763	63362
TCG	S	0.025	1.895	17751
TCT	S	0.175	13.434	125862
ACA	T	0.633	41.718	390845
ACC	T	0.041	2.724	25519
ACG	T	0.084	5.519	51702
ACT	T	0.242	15.976	149669
GTA	V	0.437	26.411	247437
GTC	V	0.131	7.925	74242
GTG	V	0.235	14.199	133023
GTT	V	0.197	11.905	111531
TGG	W	1.000	17.600	164889
TAC	Y	0.536	26.101	244527
TAT	Y	0.464	22.561	211362
TAA	*	0.600	0.001	6
TAG	*	0.000	0.000	0
TGA	*	0.400	0.000	4

[0105] Table 3: HA Influenza A H1N1 (multi) Codon Usage Preference

Coding GC 40.65%

1st letter GC 44.56%

2nd letter GC 38.20%

3rd letter GC 39.20%

Codon	AA	Fraction	Frequency	Number
GCA	A	0.467	24.211	252401
GCC	A	0.297	15.398	160530
GCG	A	0.041	2.147	22382
GCT	A	0.194	10.055	104821
TGC	C	0.407	9.531	99365
TGT	C	0.593	13.910	145017
GAC	D	0.496	24.145	251716
GAT	D	0.504	24.530	255736
GAA	E	0.686	46.936	489316
GAG	E	0.314	21.435	223463
TTC	F	0.589	19.707	205449
TTT	F	0.411	13.765	143505
GGA	G	0.361	27.066	282172
GGC	G	0.115	8.637	90040
GGG	G	0.302	22.607	235689
GGT	G	0.222	16.603	173089

CAC	H	0.469	13.712	142953
CAT	H	0.531	15.534	161951
ATA	I	0.346	20.196	210553
ATC	I	0.145	8.465	88247
ATT	I	0.509	29.734	309981
AAA	K	0.649	51.201	533784
AAG	K	0.351	27.679	288564
CTA	L	0.276	20.593	214690
CTC	L	0.121	9.003	93862
CTG	L	0.193	14.432	150461
CTT	L	0.037	2.781	28992
TTA	L	0.152	11.343	118255
TTG	L	0.220	16.445	171442
ATG	M	1.000	10.402	108443
AAC	N	0.373	29.529	307844
AAT	N	0.627	49.569	516765
CCA	P	0.489	18.306	190849
CCC	P	0.211	7.882	82167
CCG	P	0.226	8.447	88060
CCT	P	0.074	2.779	28971
CAA	Q	0.490	13.670	142513
CAG	Q	0.510	14.226	148315
AGA	R	0.740	25.010	260740
AGG	R	0.254	8.596	89617
CGA	R	0.003	0.086	898
CGC	R	0.001	0.018	191
CGG	R	0.002	0.052	540
CGT	R	0.002	0.054	564
AGC	S	0.211	16.195	168837
AGT	S	0.126	9.685	100965
TCA	S	0.376	28.927	301572
TCC	S	0.087	6.726	70119
TCG	S	0.024	1.870	19494
TCT	S	0.175	13.490	140633
ACA	T	0.626	41.367	431265
ACC	T	0.047	3.138	32717
ACG	T	0.085	5.618	58572
ACT	T	0.242	16.004	166842
GTA	V	0.432	26.042	271499
GTC	V	0.132	7.955	82929
GTG	V	0.238	14.333	149425
GTT	V	0.198	11.921	124277
TGG	W	1.000	17.632	183817
TAC	Y	0.535	26.031	271385
TAT	Y	0.465	22.638	236004
TAA	*	0.538	0.001	7
TAG	*	0.000	0.000	0
TGA	*	0.462	0.001	6

[0106] Table 4: HA Influenza A H3N2 (human) Codon Usage Preference

Coding GC 42.15%
 1st letter GC 45.23%
 2nd letter GC 39.73%
 3rd letter GC 41.49%

Codon	AA	Fraction	Frequency	Number
GCA	A	0.442	22.845	155244
GCC	A	0.223	11.563	78573
GCG	A	0.070	3.616	24574
GCT	A	0.265	13.715	93200
TGC	C	0.583	13.614	92511
TGT	C	0.417	9.736	66157
GAC	D	0.499	28.959	196792
GAT	D	0.501	29.038	197325
GAA	E	0.571	30.883	209866
GAG	E	0.429	23.205	157687
TTC	F	0.650	23.734	161285
TTT	F	0.350	12.754	86666
GGA	G	0.426	33.349	226620
GGC	G	0.151	11.798	80172
GGG	G	0.225	17.639	119865
GGT	G	0.199	15.577	105853
CAC	H	0.549	12.081	82094
CAT	H	0.451	9.925	67448
ATA	I	0.397	30.782	209179
ATC	I	0.369	28.669	194821
ATT	I	0.234	18.160	123406
AAA	K	0.752	49.034	333206
AAG	K	0.248	16.196	110057
CTA	L	0.143	9.989	67881
CTC	L	0.058	4.019	27314
CTG	L	0.279	19.411	131909
CTT	L	0.244	17.009	115585
TTA	L	0.070	4.869	33088
TTG	L	0.206	14.328	97365
ATG	M	1.000	12.013	81634
AAC	N	0.405	33.707	229053
AAT	N	0.595	49.531	336587
CCA	P	0.313	12.110	82290
CCC	P	0.205	7.938	53939
CCG	P	0.190	7.345	49912
CCT	P	0.293	11.344	77090
CAA	Q	0.745	33.793	229641
CAG	Q	0.255	11.587	78739
AGA	R	0.518	26.434	179630
AGG	R	0.292	14.905	101285
CGA	R	0.143	7.299	49598
CGC	R	0.008	0.397	2701
CGG	R	0.039	1.985	13486
CGT	R	0.000	0.011	76

AGC	S	0.315	23.589	160295
AGT	S	0.147	11.009	74808
TCA	S	0.299	22.426	152395
TCC	S	0.080	5.983	40654
TCG	S	0.002	0.121	823
TCT	S	0.157	11.795	80152
ACA	T	0.378	23.252	158007
ACC	T	0.102	6.280	42678
ACG	T	0.154	9.500	64560
ACT	T	0.366	22.543	153192
GTA	V	0.329	14.418	97977
GTC	V	0.080	3.499	23779
GTG	V	0.194	8.527	57947
GTT	V	0.397	17.436	118482
TGG	W	1.000	17.597	119580
TAC	Y	0.600	21.091	143320
TAT	Y	0.400	14.036	95379
TAA	*	0.000	0.000	0
TAG	*	0.000	0.000	0
TGA	*	1.000	0.000	1

[0107] Table 5: HA Influenza A H3N2 (multi) Codon Usage Preference

Coding GC 42.18%

1st letter GC 45.27%

2nd letter GC 39.71%

3rd letter GC 41.57%

Codon	AA	Fraction	Frequency	Number
GCA	A	0.444	22.883	169211
GCC	A	0.227	11.694	86472
GCG	A	0.068	3.506	25925
GCT	A	0.261	13.420	99235
TGC	C	0.588	13.734	101558
TGT	C	0.412	9.616	71109
GAC	D	0.508	29.541	218446
GAT	D	0.492	28.632	211725
GAA	E	0.576	31.066	229724
GAG	E	0.424	22.871	169124
TTC	F	0.653	23.655	174920
TTT	F	0.347	12.569	92946
GGA	G	0.420	32.846	242891
GGC	G	0.150	11.724	86694
GGG	G	0.228	17.797	131602
GGT	G	0.203	15.840	117134
CAC	H	0.541	11.985	88629
CAT	H	0.459	10.153	75078
ATA	I	0.396	30.534	225794
ATC	I	0.367	28.277	209105
ATT	I	0.237	18.259	135020
AAA	K	0.755	49.021	362502

AAG	K	0.245	15.906	117620
CTA	L	0.147	10.250	75799
CTC	L	0.056	3.903	28861
CTG	L	0.278	19.345	143055
CTT	L	0.242	16.827	124435
TTA	L	0.072	4.995	36940
TTG	L	0.205	14.238	105284
ATG	M	1.000	12.163	89944
AAC	N	0.410	33.995	251387
AAT	N	0.590	48.945	361939
CCA	P	0.313	12.032	88973
CCC	P	0.206	7.941	58722
CCG	P	0.190	7.293	53933
CCT	P	0.292	11.221	82974
CAA	Q	0.747	34.087	252064
CAG	Q	0.253	11.523	85207
AGA	R	0.520	26.436	195491
AGG	R	0.289	14.695	108663
CGA	R	0.136	6.933	51271
CGC	R	0.008	0.392	2901
CGG	R	0.047	2.370	17528
CGT	R	0.000	0.019	139
AGC	S	0.315	23.616	174636
AGT	S	0.148	11.080	81932
TCA	S	0.297	22.291	164840
TCC	S	0.081	6.090	45033
TCG	S	0.002	0.151	1117
TCT	S	0.157	11.800	87255
ACA	T	0.376	23.267	172056
ACC	T	0.109	6.719	49687
ACG	T	0.153	9.466	69997
ACT	T	0.363	22.451	166017
GTA	V	0.330	14.731	108929
GTC	V	0.084	3.743	27682
GTG	V	0.191	8.524	63031
GTT	V	0.394	17.579	129989
TGG	W	1.000	17.744	131210
TAC	Y	0.591	21.055	155695
TAT	Y	0.409	14.561	107675
TAA	*	0.000	0.000	0
TAG	*	0.500	0.000	1
TGA	*	0.500	0.000	1

[0108] By way of further example, exemplary protein-specific influenza codon usage preferences that have been generated by comparing influenza NA protein and nucleotide sequences are set forth below in Tables 6-10 for 1) influenza B (human), 2) influenza A H1N1 (human), 3) influenza A H1N1 (multi), 4) influenza A H3N2 (human), and 5) influenza A

H3N2 (multi), where “multi” indicates that influenza sequences from multiple animal sources (e.g., human, swine, avian) were analyzed.

[0109] Table 6: NA Influenza B (human) Codon Usage Preference

Coding GC 42.70%

1st letter GC 45.65%

2nd letter GC 47.02%

3rd letter GC 35.44%

Codon	AA	Fraction	Frequency	Number
GCA	A	0.497	32.951	122710
GCC	A	0.173	11.475	42731
GCG	A	0.029	1.928	7180
GCT	A	0.301	19.936	74241
TGC	C	0.549	21.151	78765
TGT	C	0.451	17.384	64737
GAC	D	0.376	18.458	68736
GAT	D	0.624	30.661	114180
GAA	E	0.785	42.521	158346
GAG	E	0.215	11.612	43242
TTC	F	0.272	8.577	31939
TTT	F	0.728	22.958	85494
GGA	G	0.473	45.162	168182
GGC	G	0.190	18.105	67424
GGG	G	0.234	22.381	83348
GGT	G	0.103	9.872	36762
CAC	H	0.310	7.402	27565
CAT	H	0.690	16.459	61293
ATA	I	0.524	31.908	118826
ATC	I	0.180	10.985	40909
ATT	I	0.295	17.983	66967
AAA	K	0.745	44.692	166434
AAG	K	0.255	15.260	56827
CTA	L	0.247	20.233	75349
CTC	L	0.104	8.551	31842
CTG	L	0.126	10.339	38501
CTT	L	0.118	9.712	36169
TTA	L	0.205	16.776	62473
TTG	L	0.200	16.371	60964
ATG	M	1.000	29.902	111356
AAC	N	0.492	17.205	64070
AAT	N	0.508	17.760	66137
CCA	P	0.435	21.415	79750
CCC	P	0.193	9.506	35401
CCG	P	0.111	5.490	20446
CCT	P	0.261	12.831	47781
CAA	Q	0.633	9.715	36178
CAG	Q	0.367	5.639	21000
AGA	R	0.549	24.488	91194

AGG	R	0.205	9.116	33948
CGA	R	0.141	6.281	23389
CGC	R	0.000	0.016	59
CGG	R	0.006	0.256	952
CGT	R	0.099	4.419	16455
AGC	S	0.109	8.544	31818
AGT	S	0.165	12.957	48250
TCA	S	0.425	33.319	124081
TCC	S	0.130	10.208	38016
TCG	S	0.027	2.150	8008
TCT	S	0.143	11.209	41741
ACA	T	0.477	38.419	143071
ACC	T	0.160	12.852	47861
ACG	T	0.076	6.135	22848
ACT	T	0.287	23.083	85959
GTA	V	0.266	11.492	42796
GTC	V	0.233	10.049	37421
GTG	V	0.223	9.633	35872
GTT	V	0.278	12.005	44706
TGG	W	1.000	17.130	63791
TAC	Y	0.419	17.935	66791
TAT	Y	0.581	24.903	92738
TAA	*	0.996	2.130	7931
TAG	*	0.001	0.002	7
TGA	*	0.003	0.006	24

[0110] Table 7: NA Influenza A H1N1 (human) Codon Usage Preference

Coding GC 41.92%

1st letter GC 39.38%

2nd letter GC 46.09%

3rd letter GC 40.30%

Codon AA Fraction Frequency Number

GCA	A	0.280	9.892	69837
GCC	A	0.189	6.693	47255
GCG	A	0.051	1.798	12694
GCT	A	0.480	16.973	119833
TGC	C	0.464	18.609	131382
TGT	C	0.536	21.482	151669
GAC	D	0.505	22.002	155343
GAT	D	0.495	21.599	152493
GAA	E	0.640	27.050	190980
GAG	E	0.360	15.225	107495
TTC	F	0.498	18.890	133367
TTT	F	0.502	19.063	134587
GGA	G	0.390	37.344	263659
GGC	G	0.162	15.484	109319
GGG	G	0.235	22.500	158857
GGT	G	0.213	20.363	143765
CAC	H	0.346	4.631	32694

CAT	H	0.654	8.756	61817
ATA	I	0.466	45.047	318045
ATC	I	0.220	21.316	150496
ATT	I	0.314	30.363	214368
AAA	K	0.510	21.821	154064
AAG	K	0.490	20.966	148022
CTA	L	0.260	10.196	71989
CTC	L	0.046	1.803	12728
CTG	L	0.111	4.332	30587
CTT	L	0.059	2.301	16248
TTA	L	0.236	9.252	65320
TTG	L	0.288	11.280	79642
ATG	M	1.000	15.251	107674
AAC	N	0.431	36.768	259591
AAT	N	0.569	48.628	343324
CCA	P	0.481	22.287	157349
CCC	P	0.133	6.176	43607
CCG	P	0.096	4.450	31415
CCT	P	0.289	13.393	94558
CAA	Q	0.584	17.988	126997
CAG	Q	0.416	12.817	90492
AGA	R	0.596	21.424	151262
AGG	R	0.184	6.605	46635
CGA	R	0.159	5.721	40389
CGC	R	0.050	1.781	12574
CGG	R	0.002	0.061	431
CGT	R	0.010	0.358	2529
AGC	S	0.180	20.580	145299
AGT	S	0.238	27.187	191950
TCA	S	0.249	28.412	200598
TCC	S	0.116	13.311	93981
TCG	S	0.051	5.850	41302
TCT	S	0.166	18.930	133651
ACA	T	0.390	23.049	162735
ACC	T	0.286	16.895	119286
ACG	T	0.002	0.140	990
ACT	T	0.322	19.078	134698
GTA	V	0.237	14.197	100232
GTC	V	0.203	12.153	85801
GTG	V	0.280	16.743	118207
GTT	V	0.279	16.700	117907
TGG	W	1.000	34.038	240319
TAC	Y	0.453	13.582	95894
TAT	Y	0.547	16.405	115827
TAA	*	0.865	1.740	12285
TAG	*	0.135	0.271	1914
TGA	*	0.000	0.001	7

[0111] Table 8: NA Influenza A H1N1 (multi) Codon Usage Preference

Coding GC 41.87%
 1st letter GC 39.36%
 2nd letter GC 46.11%
 3rd letter GC 40.14%

Codon	AA	Fraction	Frequency	Number
GCA	A	0.287	10.200	82144
GCC	A	0.192	6.815	54887
GCG	A	0.048	1.707	13750
GCT	A	0.473	16.826	135503
TGC	C	0.472	18.934	152483
TGT	C	0.528	21.198	170719
GAC	D	0.494	21.688	174664
GAT	D	0.506	22.259	179260
GAA	E	0.642	26.931	216883
GAG	E	0.358	15.028	121026
TTC	F	0.498	18.791	151330
TTT	F	0.502	18.971	152780
GGA	G	0.391	37.319	300546
GGC	G	0.162	15.424	124218
GGG	G	0.236	22.496	181171
GGT	G	0.212	20.222	162854
CAC	H	0.344	4.679	37683
CAT	H	0.656	8.943	72018
ATA	I	0.468	45.150	363616
ATC	I	0.217	20.912	168414
ATT	I	0.316	30.497	245602
AAA	K	0.523	22.378	180218
AAG	K	0.477	20.421	164455
CTA	L	0.247	9.793	78870
CTC	L	0.043	1.691	13619
CTG	L	0.123	4.875	39264
CTT	L	0.065	2.577	20753
TTA	L	0.244	9.689	78026
TTG	L	0.279	11.095	89352
ATG	M	1.000	15.535	125108
AAC	N	0.422	35.632	286963
AAT	N	0.578	48.871	393575
CCA	P	0.484	22.228	179014
CCC	P	0.135	6.213	50035
CCG	P	0.097	4.450	35835
CCT	P	0.284	13.021	104867
CAA	Q	0.584	17.964	144671
CAG	Q	0.416	12.773	102866
AGA	R	0.595	21.567	173685
AGG	R	0.190	6.867	55305
CGA	R	0.151	5.467	44025
CGC	R	0.046	1.660	13371
CGG	R	0.005	0.185	1490
CGT	R	0.013	0.483	3890

AGC	S	0.182	20.844	167865
AGT	S	0.239	27.294	219808
TCA	S	0.246	28.085	226184
TCC	S	0.112	12.846	103451
TCG	S	0.053	6.002	48337
TCT	S	0.168	19.213	154727
ACA	T	0.389	23.099	186022
ACC	T	0.283	16.831	135545
ACG	T	0.004	0.227	1826
ACT	T	0.324	19.268	155171
GTA	V	0.240	14.313	115270
GTC	V	0.204	12.165	97967
GTG	V	0.275	16.414	132191
GTT	V	0.281	16.772	135069
TGG	W	1.000	34.123	274810
TAC	Y	0.456	13.719	110483
TAT	Y	0.544	16.354	131703
TAA	*	0.802	1.611	12977
TAG	*	0.197	0.396	3188
TGA	*	0.001	0.002	20

[0112] Table 9: NA Influenza A H3N2 (human) Codon Usage Preference

Coding GC 42.92%

1st letter GC 42.43%

2nd letter GC 44.50%

3rd letter GC 41.84%

Codon	AA	Fraction	Frequency	Number
GCA	A	0.358	10.743	59488
GCC	A	0.213	6.392	35394
GCG	A	0.073	2.190	12126
GCT	A	0.356	10.678	59132
TGC	C	0.481	21.541	119287
TGT	C	0.519	23.276	128892
GAC	D	0.400	20.514	113598
GAT	D	0.600	30.802	170566
GAA	E	0.584	31.840	176315
GAG	E	0.416	22.692	125658
TTC	F	0.561	17.908	99167
TTT	F	0.439	14.033	77711
GGA	G	0.376	30.885	171025
GGC	G	0.192	15.755	87245
GGG	G	0.197	16.161	89493
GGT	G	0.236	19.382	107328
CAC	H	0.102	2.182	12083
CAT	H	0.898	19.201	106324
ATA	I	0.472	38.110	211036
ATC	I	0.199	16.068	88976
ATT	I	0.329	26.514	146820
AAA	K	0.628	31.235	172964

AAG	K	0.372	18.494	102411
CTA	L	0.134	7.152	39602
CTC	L	0.149	7.993	44263
CTG	L	0.151	8.075	44717
CTT	L	0.160	8.573	47473
TTA	L	0.074	3.962	21938
TTG	L	0.332	17.749	98286
ATG	M	1.000	15.054	83364
AAC	N	0.477	30.816	170643
AAT	N	0.523	33.794	187134
CCA	P	0.187	7.746	42892
CCC	P	0.207	8.603	47638
CCG	P	0.053	2.188	12118
CCT	P	0.553	22.967	127181
CAA	Q	0.664	17.015	94219
CAG	Q	0.336	8.604	47646
AGA	R	0.410	19.188	106256
AGG	R	0.349	16.316	90351
CGA	R	0.048	2.234	12373
CGC	R	0.039	1.824	10101
CGG	R	0.107	4.997	27671
CGT	R	0.048	2.240	12406
AGC	S	0.178	17.465	96714
AGT	S	0.150	14.737	81609
TCA	S	0.281	27.573	152686
TCC	S	0.241	23.651	130971
TCG	S	0.023	2.304	12759
TCT	S	0.126	12.381	68560
ACA	T	0.395	30.801	170562
ACC	T	0.285	22.251	123218
ACG	T	0.085	6.661	36883
ACT	T	0.235	18.320	101450
GTA	V	0.184	13.713	75935
GTC	V	0.176	13.149	72814
GTG	V	0.305	22.789	126193
GTT	V	0.335	25.050	138713
TGG	W	1.000	23.518	130230
TAC	Y	0.149	4.437	24571
TAT	Y	0.851	25.405	140679
TAA	*	0.994	2.100	11629
TAG	*	0.005	0.011	59
TGA	*	0.001	0.001	8

[0113] Table 10: NA Influenza A H3N2 (multi) Codon Usage Preference

Coding GC 42.89%

1st letter GC 42.41%

2nd letter GC 44.53%

3rd letter GC 41.73%

Codon AA Fraction Frequency Number

GCA	A	0.357	10.832	65702
GCC	A	0.215	6.529	39601
GCG	A	0.074	2.242	13597
GCT	A	0.353	10.715	64988
TGC	C	0.486	21.827	132388
TGT	C	0.514	23.125	140264
GAC	D	0.398	20.438	123962
GAT	D	0.602	30.881	187307
GAA	E	0.581	31.406	190490
GAG	E	0.419	22.614	137160
TTC	F	0.554	17.602	106760
TTT	F	0.446	14.164	85908
GGA	G	0.374	30.846	187090
GGC	G	0.189	15.601	94624
GGG	G	0.197	16.272	98697
GGT	G	0.239	19.697	119472
CAC	H	0.103	2.221	13469
CAT	H	0.897	19.242	116710
ATA	I	0.466	37.598	228047
ATC	I	0.202	16.296	98842
ATT	I	0.332	26.761	162317
AAA	K	0.632	31.583	191559
AAG	K	0.368	18.366	111394
CTA	L	0.138	7.375	44732
CTC	L	0.146	7.804	47337
CTG	L	0.150	8.015	48615
CTT	L	0.163	8.716	52867
TTA	L	0.079	4.216	25573
TTG	L	0.323	17.258	104675
ATG	M	1.000	15.113	91668
AAC	N	0.473	30.661	185967
AAT	N	0.527	34.140	207068
CCA	P	0.192	7.935	48128
CCC	P	0.208	8.599	52156
CCG	P	0.051	2.099	12732
CCT	P	0.549	22.718	137793
CAA	Q	0.659	16.917	102607
CAG	Q	0.341	8.755	53103
AGA	R	0.410	19.203	116470
AGG	R	0.353	16.527	100239
CGA	R	0.051	2.414	14643
CGC	R	0.039	1.849	11216
CGG	R	0.103	4.838	29345
CGT	R	0.044	2.050	12433
AGC	S	0.178	17.426	105693
AGT	S	0.152	14.883	90273
TCA	S	0.278	27.261	165347
TCC	S	0.241	23.553	142859
TCG	S	0.025	2.466	14956
TCT	S	0.126	12.341	74850

ACA	T	0.395	30.737	186433
ACC	T	0.283	22.045	133712
ACG	T	0.084	6.501	39428
ACT	T	0.238	18.495	112178
GTA	V	0.191	14.196	86104
GTC	V	0.176	13.096	79434
GTG	V	0.300	22.375	135712
GTT	V	0.333	24.837	150648
TGG	W	1.000	23.690	143689
TAC	Y	0.154	4.620	28023
TAT	Y	0.846	25.306	153489
TAA	*	0.994	2.099	12729
TAG	*	0.005	0.011	65
TGA	*	0.001	0.002	11

[0114] Thus, in certain embodiments of the methods described herein, the optimized nucleotide sequence encoding for an engineered HA influenza protein is generated using the HA-specific influenza codon usage preferences set forth in one of Tables 1-5. In some embodiments of the methods described herein, the optimized nucleotide sequence encoding for an engineered NA influenza protein is generated using the NA-specific influenza codon usage preferences set forth in one of Tables 6-10.

Further Optimization by Modifying Other Regions of Structural Influenza Protein

[0115] In addition to changing codons, the optimized nucleotide sequences encoding the engineered influenza structural protein can optionally be further optimized through the modification of the sequence by i) using 5' - and/or 3' non-coding sequences from the structural proteins of wild type or other recovered viruses, such as a high titer, recovered virus; and/or ii) using 5' and 3' terminal coding sequences, encoding signal peptide, transmembrane domains, and/or cytoplasmic tails from wild type or other recovered viruses, such as high titer, recovered virus. *See e.g.*, Harvey et al.(2011), J. Virol.85(12):6086-6090; Gomila et al. (2013), Vaccine (31():4736-4743. By way of example, these additional modifications are depicted in Steps 4 and 5 of Figure 1. Each of the modifications may be applied to the optimized nucleotide sequences independently or in combination and does not modify the ectodomain (extracellular) coding portion of the protein.

[0116] Identifying the 5' and/or 3' non-coding regions, signal peptides, transmembrane domains, cytoplasmic domains, and/or ectodomains of proteins, such as structural influenza proteins, is routine in the art and can be carried out using known methods and techniques.

[0117] For example, the location of the signal peptide and ectodomain sequences of structural influenza proteins, such as HA, can be determined based on sequence alignments and reference to influenza A subtype H1N1 and H3N2 structural models in RCSB PDB, which are available through the world wide web at rcsb.org. The signal peptide can also be determined through the use of software for prediction the signal peptides, such as SignalP (Thomas Nordahl Petersen et al., *Nature Methods*, 8:785-86, 2011).

[0118] The signal peptide of influenza A subtype H1N1 encompasses residues 1-17 of the H1N1 polypeptide. The ectodomain starts with the residue D at position 18. An annotated alignment of H1N1 HA protein sequences is shown in Figure 2. Commonly, the ectodomain sequence begins with DTIC (SEQ ID NO: 19) for seasonal-like sequences or DTLT (SEQ ID NO: 20) for pandemic like sequences. H.M. Berman et al., *The Protein Data Bank. Nucleic Acids Research*, 28: 235-242, 2000. The signal peptide of influenza A subtype H3N2 encompasses residues 1-16 of the H3N2 polypeptide. The ectodomain starts with the residue Q at position 17. An annotated alignment of H3N2 HA protein sequences is shown in Figure 3. Commonly, the ectodomain sequence begins with QKLP (SEQ ID NO: 21) or QDLP (SEQ ID NO: 22). H.M. Berman et al., *The Protein Data Bank. Nucleic Acids Research*, 28: 235-242, 2000.

[0119] Similarly, the location of the transmembrane and cytoplasmic domain sequences of structural influenza proteins, such as HA, can be determined based on sequence alignments. The sequence alignment of the HA transmembrane domain of various representative influenza A strains is shown in Figure 4. See also, *Secondary Structure, Orientation, Oligomerization, and Lipid Interactions of the Transmembrane Domain of Influenza Hemagglutinin*. Suren A. Tatulian and Lukas K. Tamm. *Biochemistry*, 2000, 39 (3), pp 496–507. Software is also available for the skilled artisan to identify transmembrane and cytoplasmic domains, including, for example, TMPred (K. Hofmann & W. Stoffel, 1993, *TMbase - A database of membrane spanning proteins segments*. *Biol. Chem. Hoppe-Seyler* 374,166); InterProScan (Zdobnov E.M. and Apweiler R., 2001, *Bioinformatics*, 17(9): 847-48); and TMHMM (Krogh, B. et al., *Journal of Molecular Biology*, 2001, 305(3):567-580).

[0120] Thus, in certain embodiments, the methods of generating an optimized nucleotide sequence encoding an engineered influenza structural protein, further comprises one or more of the following steps:

- a) adding 5' and 3' non-coding sequences from another influenza strain, such as a high titer rescued strain;
- b) exchanging the sequence encoding the signal peptide in the optimized nucleotide sequence with a nucleotide sequence encoding the signal peptide from another influenza strain, such as a high titer rescued strain;
- c) exchanging the sequence encoding the transmembrane domain in the optimized nucleotide sequence with a nucleotide sequence encoding the transmembrane from another influenza strain, such as a high titer rescued strain; and/or
- d) exchanging the sequence encoding the cytoplasmic domain in the optimized nucleotide sequence with a nucleotide sequence encoding the cytoplasmic domain from another influenza strain, such as a high titer rescued strain.

[0121] In certain embodiments, the methods described herein further comprise step a); step b); step c); step d); steps a) and b); steps a) and c); steps a) and d); steps a), b), and c); steps a), b), and d); steps a), c), and d); steps a), b), c), and d); steps b) and c); steps b) and d); steps b), c), and d); or steps c) and d).

[0122] The 5' and 3' non-coding sequences from another influenza strain can further comprise coding sequence without disrupting the amino acid sequence. Thus, the 5' and 3' terminal nucleotide sequences can include non-coding and coding sequences. In some embodiments, the 5' and 3' terminal sequences are predominantly coding sequence, including the signal peptide and extending into the stem region at the 5' end; and including the stem, transmembrane region and cytoplasmic tail at the 3' end.

Optimized Nucleotide Sequence Encoding an Engineered Influenza Structural Protein

[0123] Another aspect is directed to an optimized nucleotide sequence encoding the engineered influenza structural protein that is obtained by the methods described herein, wherein at every position where the codons in the reverse translated nucleotide sequence (i.e., the first nucleotide sequence) and a second nucleotide sequences (that encodes a corresponding influenza structural protein from a wild type virus or a previously rescued virus) code for the same amino acid, the codons in the optimized nucleotide sequence have been changed to match the codons from the second nucleotide sequence; and wherein at every position where the codons in the first and second nucleotide sequences code for a different amino acid, the codons in the

optimized nucleotide sequence have been changed to match codons that are based on influenza protein-specific influenza codon usage preferences.

[0124] In certain embodiments, the optimized nucleotide sequence further comprises one or more of the following modifications:

a) 5' and 3' non-coding nucleotide sequences (e.g., non-coding sequences) from another influenza strain, such as a high titer rescued strain;

b) a nucleotide sequence encoding the signal peptide from another influenza strain, such as a high titer rescued strain;

c) a nucleotide sequence encoding the transmembrane from another influenza strain, such as a high titer rescued strain; and/or

d) a nucleotide sequence encoding the cytoplasmic domain from another influenza strain, such as a high titer rescued strain.

[0125] In certain embodiments, the optimized nucleotide sequence further comprises modification a); modification b); modification c); modification d); modifications a) and b); modifications a) and c); modifications a) and d); steps a), b), and c); modifications a), b), and d); modifications a), c), and d); modifications a), b), c), and d); modifications b) and c); modifications b) and d); modifications b), c), and d); or modifications c) and d).

Engineered Influenza Proteins

[0126] The methods described herein for optimizing nucleotide sequences are preferably performed on engineered influenza structural proteins, including, but not limited to, HA and NA. The methods described herein can be performed on any engineered influenza structural protein.

[0127] For example, to induce more broadly reactive immune responses, computationally optimized broadly reactive antigens (COBRAs) have been developed for influenza HA proteins through a series of HA protein alignments and subsequent consensus sequences based on selected H5N1 and H1N1 influenza virus isolates, as described in WO2013/122827 and US Publication Nos. 2015/0044247, 2015/0017196, 2014/0147459, 2014/0127248, and 2013/0183342, all of which are hereby incorporated by reference in their entirety.

[0128] These recombinantly engineered COBRAs have a uniquely designed amino acid sequence for eliciting a broadly reactive immune response against a broad range of influenza isolates, such as most or all influenza viruses within a specific subtype, such as H1N1 or H5N1. The amino acid sequence of the COBRAs does not occur in nature. In addition to the specific COBRAs described in WO2013/122827 and US Publication Nos. 2015/0044247, 2015/0017196, 2014/0147459, 2014/0127248, and 2013/0183342, it is also possible to generate other recombinantly engineered COBRAs using the methods disclosed in these published applications.

[0129] The amino acid sequences of certain exemplary H5N1 COBRAs are set forth in Table 11.

[0130] Table 11: Exemplary H5N1 COBRA Amino Acid Sequences

<p>All H5N1 COBRA</p> <p>MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTTHAQDILEKTHN GKLCDLDDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKASPANDLCY PGDFNDYEELKHLLSRINHFEEKIIPKSSWSNHEASSGVSSACPYQGKSSFFRN VWLIKKNSAYPTIKRSYNNNTNQEDLLVLWGIHHPNDAAEQTKLYQNPTTYIS VGTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAP EYA YKIVKKGDS AIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGEC PKYVK SNRLVLATGLRNSPQRERRRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNE QSGGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNK KMEDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKEL GNGCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGTYQIL SIYSTVASSLALAIMVAGLSLWMCSNGSLQCRICI</p> <p>(SEQ ID NO: 1)</p>
<p>Human/Avian COBRA-2</p> <p>MEKIVLLLAIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTTHAQDILEKTHN GKLCDLDDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPANDLCY PGNFNDYEELKHLLSRINHFEEKIIPKSSWSDHEASSGVSSACPYQGKSSFFRN VWLIKKNSAYPTIKRSYNNNTNQEDLLVLWGIHHPNDAAEQTRL YQNPTTYISV GTSTLNQRLVPKIATRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAP EYAY KIVKKGDS AIMKSELEYGNCNTKCQTPMGAINSSMPFHNIHPLTIGEC PKYVKS NRLVLATGLRNSPQRERRRRKKRGLFGAIAGFIEGGWQGMVDGWYGYHHSNEQG SGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKM EDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDNAKELGN GCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGTYQILSIY STVASSLALAIMVAGLSLWMCSNGSLQCRICI</p> <p>(SEQ ID NO: 2)</p>

Human COBRA-2

MEKIVLLLAIIVSLVKSDQICIGYHANNSTEQVDTIMEKNVTVTTHAQDILEKTHN
GKLCDLDGVKPLILRDCSVAGWLLGNPMCDEFINVPEWSYIVEKANPANDLCY
PGNFNDYEELKHLLSRINHFEEKIIPKSSWSDHEASSGVSSACPYQGSPSFFRNV
VWLIKKNNTYPTIKRSYNNTNQEDLLVLWGIHHPNDAAEQTRL YQNPTTYISV
GTSTLNQRLVPKIA TRSKVNGQSGRMEFFWTILKPNDAINFESNGNFIAP EYAY
KIVKKGDS AIMKSELEYGNCNTKCQTPIGAINSSMPFHNIHPLTIGEC PKYVKSN
RLVLATGLRNSPQRESRRKKRGLFGA IAGFIEGGWQGMVDGWYGYHHSNEQG
SGYAADKESTQKAIDGVTNKVNSIIDKMNTQFEAVGREFNNLERRIENLNKKM
EDGFLDVWTYNAELLVLMENERTLDFHDSNVKNLYDKVRLQLRDN AKELGN
GCFEFYHKCDNECMESVRNGTYDYPQYSEEARLKREEISGVKLESIGTYQILSIY
STVASSLALAIMVAGLSLWMCSNGSLQCRICI

(SEQ ID NO: 3)

[0131] The amino acid sequences of certain exemplary H1N1 COBRAs are set forth in Table 12.

[0132] Table 12: Exemplary H1N1 COBRA Amino Acid Sequences

Pandemic H1N1 COBRA (Human and Swine 1933-2011): P1

MKARLLVLLCALAATDADTICIGYHANNSTD TVDTVLEKNVTVTTHSVNLLED SH
NGKLCKLKGIAPLQLGKCNIAGWLLGNPECESLLSARSWSYIVETPNSENGTCYP
GDFIDYEELREQLSSVSSFERFEIFPKESSWPNHNTTKGVTAACSHAGKSSFYRNL
LWLTKKGGSYPKLSKSYVNNKGKEVLVLWGVHHPSTSTDQQSL YQENAYVS
VVSSNYNRRFTPEIAERP KVRGQAGRMNYYWTLLEPGDTIIFEATGNLIAPWYAF
ALSRGSGSGIITSNASMHECNTKCQTPQGAINSSLPFQNIHPVTIGEC PKYVRSTKL
RMVTGLRNIPSISQSRGLFGA IAGFIEGGWTGMIDGWYGYHHQNEQGS GYAADQ
KSTQNAINGITNKVNSVIEKMNTQFTA VGKEFN NLEKRMENLNKKVDDGFLDIW
TYNAELLV LLENERTLDFHDSNVKNLYEKVKSQLRNNAKEIGNGC FEFYHKCDN
ECMESVKNGT YDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSL VLLV
SLGAISFWMC SNGSLQCRICI

(SEQ ID NO: 4)

Seasonal H1N1 COBRA (Human 1999-2012): X6

MEARLLVLLCAFAATNADTICIGYHANNSTD TVDTVLEKNVTVTTHSVNLLED SH
NGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENGT CYP
GYFADYEELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLL
WLTGKNGLYPNLSKSYANNKEKEVLVLWGVHHPNIGDQRAL YHTENAYVSV
VSSHYSRKFTPEIAKRPKVRDQEGRINYWTLLEPGDTIIFEANGNLIAPRYAFAL
SRGFGSGIITSNAPMDECDACQTPQGAINSSLPFQNVHPVTIGEC PKYVRS AKLR
MVTGLRNIPSISQSRGLFGA IAGFIEGGWTGMVDGWYGYHHQNEQGS GYAADQK
STQNAINGITNKVNSVIEKMNTQFTA VGKEFN KLERRMENLNKKVDDGFLDIWT
YNAELLV LLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGC FEFYHKCNN

ECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLV
SLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 5)

Seasonal H1N1 COBRA (Human 1978-2008): X3

MEARLLVLLCAFAATNADTICIGYHANNSTDVDTVLEKNVTVTTHSVNLLED SH
NGKLCRLKGIAPLQLGNCSVAGWILGNPECESLFSKESWSYIAETPNPENGTCYP
GYFADYEELREQLSSVSSFERFEIFPKESSWPNNHTVTKGVTASCSHNGKSSFYRNL
LWLTEKNGLYPNLSKSYVNNKEKEVLVLWGVHHPNSNIGDQRAIYHTENAYVSV
VSSHYSRRFTPEIAKRPKVRDQEGRINYWTLLPEGDTIIFEANGNLIAPWYAFAL
SRGFGSGIITSNASMDECDACQTPQGAINSSLPFQNVHPVTIGECPKYVRSTKLR
MVTGLRNIPSIQSRGLFGAIAAGFIEGGWTGMIDGWYGYHHQNEQSGYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTY
NAELLVLENERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCNNEC
MESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSL
GAISFWMCNGLSLQCRICI

(SEQ ID NO: 6)

Seasonal H1N1 COBRA (Human 1918-2012): X1

MEARLLVLLCAFAATNADTICIGYHANNSTDVDTVLEKNVTVTTHSVNLLED SH
NGKLCCKLKGIAPLQLGKCNIAWILGNPECESLLSKRSWSYIVETPNSENGTCYP
GDFIDYEELREQLSSVSSFERFEIFPKESSWPNNHTTKGVTAACSHAGKSSFYRNL
LWLTKKNGSYPNLSKSYVNNKGKEVLVLWGVHHPNSNIEDQQSLYQENAYVSV
VSSNYNRRFTPEIAKRPKVRDQEGRMNYWTLLPEGDTIIFEANGNLIAPWYAFAL
LSRGFGSGIITSNASMHECDTKCQTPQGAINSSLPFQNIHPVTIGECPKYVRSTKLR
MVTGLRNIPSIQSRGLFGAIAAGFIEGGWTGMIDGWYGYHHQNEQSGYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNLEKRMENLNKKVDDGFLDIWT
YNAELLVLENERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCNN
ECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLV
SLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 7)

H1N1 COBRA (1918-2011): A1

MKAKLLVLLCAFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNLLLED SH
NGKLCRLKGIAPLQLGNCSIAGWILGNPECESLFSKESWSYIVETPNSENGTCYPG
YFADYEELREQLSSVSSFERFEIFPKESSWPNHNTVTKGVTASCSHNGKSSFYRNLL
WLTEKNGSYPNLSKSYVNNKEKEVLVLWGVHHPNSNIGDQRAIYHTENAYVSVV
SSHYSRRFTPEIAKRPKVRDQEGRINYWTLLPEPGDTIIFEANGNLIAPWYAFALS
RGFGSGIITSNASMDECDACQTPQGAINSSLPFQNVHPVTIGECPKYVRSTKLR
MVTGLRNIPSIQSRGLFGAIAFGFIEGGWTGMIDGWYGYHHQNEQSGYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWY
NAELLVLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCNNEC
MESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSL
GAISFWMCSNGSLQCRICI

(SEQ ID NO: 8)

[0133] In some embodiments, an engineered COBRA has a sequence at least about 90% (e.g., at least about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) identical to a sequence that appears in Table 11 or 12. In some embodiments, an engineered HA COBRA has a sequence that is substantially identical to a sequence that appears in Table 11 or 12. In some embodiments, an engineered HA COBRA has a sequence that is identical to a sequence that appears in Table 11 or 12.

[0134] By way of further example, engineered HA sequences have been developed using a rational design approach to include epitopes from multiple viral isolates in a polyvalent vaccine, as described in PCT/US2016/035594 (claiming priority to U.S. Provisional Application No. 62/169,814), which is hereby incorporated by reference in its entirety. In certain embodiments, the designs are based on combinations of multiple B cell epitopes and antigenic regions from different HA sequences (subtype H1) into mosaic antigens. These mosaic epitope antigens, in some embodiments, are predicted to confer cross-protection against multiple subtype H1 strains by maximizing sequence homology for at least one neutralizing epitope. The best mosaic sequence templates are selected by evaluating overall alignment coverage by geographic regions, viral isolate years, sequence clusters or other scoring methods. The selected set of mosaic template sequences are combined with target backbone sequences to generate a set of full-length mosaic protein sequences. Structure refinement of these mosaic sequences yields the final set of vaccination proteins. The amino acid sequences of these engineered HA proteins do not match the amino acid sequences of any naturally occurring strains. The amino acid sequences of certain exemplary engineered HA proteins are set forth in Table 13.

[0135] Table 13: Exemplary H1N1 Mosaic HA Proteins

<p>SP1</p> <p>MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SH NGKLCKLKGIAPLQLGKCSVAGWILGNPECESLSTASSWSYIVETSNPENGTCYP GYFADYEELREQLSSVSSFERFEIFPKESSWPNHVTGTASCSHAGKSSFYRNLL WLTGKNGSYPNLSKSYVNNKEKEVLVLWGVBHPSNIGDQQTLYQTENAYVSV VSSRYRRTPEIAKRPKVRDQEGRMNYYWTLVEPGDTIIFEANGNLIAPWYAFAL LSRGFGSGIITSNAPVHDCNTKCQTPQGAINSSLPFQNVHPVTIGECPKYVRSACL RMTATGLRNIPSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADQ KSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIW TYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFFFYHKCN NTCMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLL VSLGAISFWMCSNGSLQCRICI</p> <p>(SEQ ID NO: 9)</p>
<p>SP2</p> <p>MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SH NGKLCLLKGIAPLQLGNCSVAGWILGNPECELLSTKSSWSYIVETPNPENGTCYP GYFADYEELREQLSSVSSFERFEIFPKESSWPNHDTGVSASCSHNGASSFYRNLL WLTCKNNLYPNLSKSYANNKGKEVLVLWGVBHHPSTIADQQTLYHTENAYVSV VSSHYSRRTPEIAIRPKVRDQEGRINYWTLLEPGDTIIFEANGNLIAPWYAFAL SRGFSGGIITSNAPMDECNTTCQTPQGAINSSLPFQNVHPVTIGECPKYVRSACKL MVTGLRNIPSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADLK STQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWT YNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFFFYHKCN ECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLV SLGAISFWMCSNGSLQCRICI</p> <p>(SEQ ID NO: 10)</p>
<p>SP3</p> <p>MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SH NGKLCKLKGIAPLQLGKCSVAGWILGNPECESLSTASSWSYIVETSSPDNGTCYP GYFADYEELREQLSSVSSFERFEIFPKTSSWPNHDSNGVTASCPHAGAKSFYRNLL LWLVKKGNSYPKLSKSYINDKGKEVLVLWGVBHHPSTSADQQSLYQNANAYVS VVTSRYRRTPEIAIRPKVRDQEGRMNYYWTLVEPGDTIIFEATGNLIAPWYAFAL LSRGFGSGIITSNAPVHDCNTTCQTPQGAINSSLPFQNVHPVTIGECPKYVRSACKL RMTATGLRNIPSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADL LKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDI WTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFFFYHKCN NNTCMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLV SLGAISFWMCSNGSLQCRICI</p> <p>(SEQ ID NO: 11)</p>

SP4

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILED SH
NGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYP
GYFADYEELREQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLL
WLTGKNGLYPNLSKSYANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSV
VSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL
SRGFGSGIITSNAPMDKCDACQTPQGAINSSLPFQNVHPVTIGECPKYVRS AKLR
MVTGLRNIPFIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADQK
STQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWT
YNAELLVLENERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCND
ECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLV
SLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 12)

SP5

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILED SH
NGKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYP
GYFADYEELREQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCPHNGESSFYRNLL
WLTGKNGLYPNLSKSYANNKEKEVLVLWGVHHPNIGDQKTLYHTENAYVSV
VSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFAL
SRGFGSGIITSNAPMDKCDACQTPQGAINSSLPFQNVHPVTIGECPKYVRS AKLR
MATGLRNIIQSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADLK
STQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWT
YNAELLVLENERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCNN
TCMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLV
SLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 13)

SP6

MKAILVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTTHSVNLLEDK
HNGKLCKLRGVAPLHLGKCNIAGWILGNPECESLSTASSWSYIVETSNSENGTCY
PGDFIDYEELREQLSSVSSFERFEIFPKESSWPNHVTGKVTAAACSHAGKSSFYKN
LIWLTGKNGSYPNLSKSYVNNKEKEVLVLWGIHHPNIGDQQTL YQTEDTYV FV
GSSRYSKKFKPEIAKRPKVRDQEGRMNYWTLVEPGDKITFEANGNLVVP RYAF
AMERNAGSGIIISNAPVHDCNTKCQTPKGAINSSLPFQNIHPITIGKCPKYVKSTKL
RLATGLRNIPSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADQ
KSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWT
YNAELLVLENERTLDYHDSNVKNLYEKVRSQKNNAKEIGNGCFEFYHKCDN
TCMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVLVVS
LGAISFWMCSNGSLQCRICI

(SEQ ID NO: 14)

SP7

MKAILVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDK
HNGKLCLLRGVAPLHLGNCNIAGWILGNPECELLSTKSSWSYIVETPNSENGTCY
PGDFIDYEELREQLSSVSSFERFEIFPKESSWPNHDVTKGVSAACSHNGASSFYKN
LIWLTKKNNLYPNLSKSYANNKGKEVLVLWGIHHPSTIADQQTLYHTEDTYVVFV
GSSHYSKKFKPEIAIRPKVRDQEGRINYWTLLEPGDKITFEANGNLVVPYAF
MERNAGSGIIISNAPMDECNTTCQTPKGAINSTLFPQNIHPITIGKCPKYVKSTKL
LVTGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSYAADLKS
TQNAINEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWTY
NAELLVLENERTLDYHDSNVKNLYEKVRSQKNNAKEIGNGCFEFYHKCDNE
CMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVLVSL
GAISFWMCNGLSLQCRICI

(SEQ ID NO: 15)

SP8

MKAILVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDK
HNGKLCKLRGVAPLHLGKNCNIAGWILGNPECESLSTASSWSYIVETSSSDNGTCY
PGDFIDYEELREQLSSVSSFERFEIFPKTSSWPNHDSNKGVTAAACPHAGAKSFYKN
LIWL VKKGNSYPKLSKSYINDKGKEVLVLWGIHHPSTSADQQSLYQNADTYVVFV
GTSRYSKKFKPEIAIRPKVRDQEGRMNYYWTLVEPGDKITFEATGNLVVPYAF
AMERNAGSGIIISDTPVHDCNTTCQTPKGAINSTLFPQNIHPITIGKCPKYVKSTKL
RLATGLRNIPSIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSYAADL
KSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWT
YNAELLVLENERTLDYHDSNVKNLYEKVRSQKNNAKEIGNGCFEFYHKCDN
TCMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVLVVS
LGAISFWMCNGLSLQCRICI

(SEQ ID NO: 16)

SP9

MKAILVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNILEDKH
NGKLCLLRGVAPLHLGNCNIAGWILGNPECELLISKESWSYIVEKPNSENGTCYP
GDFIDYEELREQLSSVSSFERFEIFPKESSWPNHTVTKGVSAACSHNGKSSFYKNL
IWL TGKNGLYPNLSKSYANNKEKEVLVLWGIHHPNIGDQRALYHTEDTYVVFV
SSHYSKKFKPEIAKRPKVRDQEGRINYWTLLEPGDKITFEANGNLVVPYAF
MERNAGSGIIISNAPMDKCDKACQTPKGAINSTLFPQNIHPITIGKCPKYVKSTKL
RLVTGLRNIPFIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSYAADQ
KSTQNAINEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWT
YNAELLVLENERTLDYHDSNVKNLYEKVRSQKNNAKEIGNGCFEFYHKCDD
ECMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVLVVS
LGAISFWMCNGLSLQCRICI

(SEQ ID NO: 17)

SP10

MKAILVLLYTFATANADTLCIGYHANNSTDTVDTVLEKNVTVTTHSVNILEDKH
NGKLCLLRGVAPLHLGNCNIAGWILGNPECELLISKESWSYIVEKPNSENGTCYP
GDFIDYEELREQLSSVSSFERFEIFPKESSWPNHTVTKGVSAACPHNGESSFYKNLI
WLTGKNGLYPNLSKSYANNKEKEVLVLWGIHHPNIGDQKTLYHTEDTYVFG
SSHYSKKFKPEIAKRPKVRDQEGRINYWTLLEPGDKITFEANGNLVVPYAF
MERNAGSGIIISNAPMDKCDAKCQTPKGAINSTLPPQNIHPITIGKCPKYVKSTKL
RLATGLRNIQSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQSGSYAADL
KSTQNAINEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGFLDIWT
YNAELLVLENERTLDYHDSNVKNLYEKVRSQKNNAKEIGNGCFEFYHKCDN
TCMESVKNGTYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVASSLVVVS
LGAISFWMCNSGLQCRICI

(SEQ ID NO: 18)

[0136] By way of further example, engineered HA sequences from influenza B have been developed using a rational design approach, as described in U.S. Provisional Application No. 62/344,862), which is hereby incorporated by reference in its entirety. The amino acid sequences of these engineered HA proteins do not match the amino acid sequences of any naturally occurring strains. The amino acid sequences of certain exemplary engineered influenza B HA proteins are set forth in Table 14.

[0137] Table 14: Exemplary Influenza B SMARt HA Proteins

br08_CO1

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKATQGEVNVTVGVIPLTTTPTKSHFANLKG
TETRGKLCPKCLNCTDLDAVALGRPKCTGKIPSARVSILHEVRPVTSGCFPIIMHDRTKIRQL
PNLLRGYEHIRLSTQNVINAENAPGGPYKIGTSGSCP NATNKS GFFATMAWAVPKNDNN
KTATNPLTIEVPYICTEGEDQITVWGFHSDNKTQMKKLYGDSKPQKFTSSANGVTTHYV
SQIGGFPNQTEDGGLPQSGRIVVDYMVQKPGKTGTIVYQRGILLPQKVWCASGRSKVIK
GSLPLIGEADCLHEKYGGLNKSPPYYTGEHAKAIGNCPIWVKTPKLANGTKYRPPAKL
LKERGFFGAIAAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNS
LSELEVKNLQRLSGAMDELHNEILELDEKVDDL RADTISSQIELAVLLSNEGIINSEDEHL
LALERKLLKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEFSLPTFDSL NITAA
SLNDDGLDNHTILLYYSTAASSLAVTLMIAIFVVYMVSRDNVSCSICL

(SEQ ID NO: 75)

br08_DO2

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKATATQGEVNVTVGIPLTTTPTKSYFANLKG
TETRGLKCPKCLNCTDLDVALGRPKCTGKIPSAKVSILHEVRPVTSGCFPIIMHDRTKIRQL
PNLLRGYEHIRLSTQNVIDAENAPGGPYKIGTSGSCP NATNKS GFFATMAWAVPKNDNN
KTATNPLTIEVPYICTEGEDQITVWGFHSDNKTQMKKLYGDSKPQKFTSSANGVTTHYV
SQIGGFPDQTEDGGLPQSGRIVVDYMVQKPGKTGTIVYQRGILLPQKVWCASGRSKVIK
GSLPLIGEADCLHEKYGGLNKS KPYTGEHAKAIGNCPIWVK TPLKLANGTKYRPPAKL
LKERGFFGAIAGFLEGGWEGMVAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNS
LSELEIKNLQRLSGAMDELHNEILELDEKVDDL RADTISSQIELAVLLSNEGIINSEDEHLL
ALERKLLKKMLGPSAVEIGNGCFETKHKCNQTCLDRIAAGTFDAGEFSLPTFDSL NITAAS
LNDDGLDNHTILLYYSTAASSLAVTLMIAIFVVYMVSRDNVSCSICL

(SEQ ID NO: 76)

br08_DO3

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKATATQGEVNVTVGISLTTTPTKSHFANLKG
TKTRGKLCPCPNCTDLDVALGRPMCTGTIPSAKVSILHEVRPVTSGCFPIIMHDRTKIRQL
PNLLRGYEHIRLSTHN VINAENAPGGPYKIGTSGSCP NATNKG FFFATMAWAVPKNDNN
KTATNPLTIEVPYICAEGEDQITVWGFHSDDKTQMKKLYGDSKPQKFTSSANGVTTHYV
SQIGDFPNQTEDGGLPQSGRIVVDYMVQKPGKTGTITYQRGILLPQKVWCASGRSKVIK
SLPLIGEADCLHEKYGGLNKS KPYTGEHAKAIGNCPIWVK TPLKLANGTKYRPPKLL
KERGFFGAIAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNSL
SELEVKNLQRLSGAMDELHNEILELDEKVDDL RADTISSQIELAVLLSNEGIINSEDEHLL
ALERKLLKKMLGPSAVEIGNGCFETKHKCNQTCLNRIAAGTFDAGEFSLPTFDSL NITAAS
LNDDGLDNHTILLYYSTAASSLAVTLMIAIFVVYMVSRDNVSCSICL

(SEQ ID NO: 77)

pan90_DO2

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKATATQGEVNVTVGIPLTTTPTKSYFANLKG
TETRGLKCPNCLNCTDLDVALGRPKCVGKIPSAKASILHEVRPVTSGCFPIIMHDRTKIRQL
PNLLRGYEHIRLSTQNVIDAERAPGGPYRLGTSGSCP NATSKS GFFATMAWAVPKDDNN
KTATNPLTVEVPYICTEGEDQITVWGFHSDNKTQMKNLYGDSNPQKFTSSANGVTTHYV
SQIGGFPDQTEDGGLPQSGRIVVDYMVQKPGKTGTIVYQRGVLLPQKVWCASGRSKVIK
GSLPLIGEADCLHEKYGGLNKS KPYTGEHAKAIGNCPIWVK TPLKLANGTKYRPPAKL
LKERGFFGAIAGFLEGGWEGMVAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNS
LSELEIKNLQRLSGAMDELHNEILELDEKVDDL RADTISSQIELAVLLSNEGIINSEDEHLL
ALERKLLKKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSL NITAAS
LNDDGLDNHTILLYYSTAASSLAVTLMIAIFIVYMVSRDNVSCSICL

(SEQ ID NO: 78)

ma12_RA82

MKAIIVLLMVVTSNADRICTGITSSKSPHVVKATATQGEVNVTVGIPLTTTPTKSHFANLRG
TKTRGKLCPCDCLNCTDLVALGRPKCVGNTPSAKASILHEVRPVTSGCFPIMHDRTKIRQ
LANLLRGYEHIRLSNYNVIDAEKAPGGPYRLGTSRSCPNVTSRSGFFATMAWAVPKDDN
NKNATNPLTVEVPYICTEGEDQITVWGFHSDNKTQMVNLYGDSNPQKFTSSANGVTTH
YVSQIGDFPNQTEDGGLPQSGRIVVDYMMQKSGKTGTITYQRGVLLPQKVWCASGRSK
VIKGTPLIGEADCLHEKYGGLNKSPPYYTGEHAKAIGNCPIWVKTPKLANGTKYRPP
AKLLKERGFFGAIAAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKN
LNSLSELEVKNLQRLSGAMDELHNEILELDEKVDDLADTSSQIELAVLLSNEGIINSEDE
HLLALERKLLKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNT
AASLNDGDGLDNHTILLYSTAASSLAVTLMIAIFIVYMSRDNVSCSICL

(SEQ ID NO: 79)

sing79_RA103

MKAIIVLLMVVTSNADRICTGITSSNSPHVVKATATQGEVNVTVGIPLTTTPTKSYFANLKG
TKTRGKLCPCNCLNCTDLVALGRPMCMGTIPSAKASILHEVRPVTSGCFPIMHDRTKIRQ
LPNLLRGYENIRLSTHNINAERAPGGPYIIGTSGSCPNATNKNNGFFATMAWAVPKDDNN
KTATNPLTVEVPYICTEGEDQITVWGFHSDNKTQMKKLYGDSKPQKFTSSANGVTTHYV
SQIGGFPDQTEDGGLPQSGRIVVDYMVQKSGKTGTITYQRGVLLPQKVWCASGRSKVIK
GSLPLIGEADCLHEKYGGLNKSPPYYTGEHAKAIGNCPIWVKTPKLANGTKYRPPAKL
LKERGFFGAIAAGFLEGGWEGMIAGWHGYTSHGAHGVAVAADLKSTQEAINKITKNLNS
LSELEVKNLQRLSGAMDELHNEILELDEKVDDLADTSSQIELAVLLSNEGIINSEDEHL
LALERKLLKMLGPSAVDIGNGCFETKHKCNQTCLDRIAAGTFNAGEFSLPTFDSLNTAA
SLNDGDGLDNHTILLYSTAASSLAVTLMIAIFIVYMSRDNVSCSICL

(SEQ ID NO:80)

[0138] By way of further example, engineered HA sequences have been developed to extend a seasonal response profile to cover pandemic strains, or vice versa as described in U.S. Provisional Application No. 62/354,502, which is hereby incorporated by reference in its entirety. These strategies extend the immune profile across clusters of sequences (or clades) of antigenically distinct strains; they can be applied to an engineered recombinant HA molecule over time so that it continues to elicit an immune response against antigenically drifted circulating seasonal strains. The strategy is designed to generally preserve specific residues of the receptor binding site (RBS) of a host HA polypeptide with modifications engineered in the region near the RBS. Similar strategies may be used to extend a pandemic response profile to cover seasonal strains. The modifications described in U.S. Provisional Application No. 62/354,502, can be used to further tailor or optimize the immunogenic profile so that an engineered HA polypeptide is re-engineered to elicit antibodies against more or less seasonal strains (or demonstrate an improved or more anti-seasonal antibody response) or more or less pandemic strains (or demonstrate an improved or more anti-pandemic antibody response). The

amino acid sequences of these modified, engineered HA proteins do not match the amino acid sequences of any naturally occurring strains. The amino acid sequences of certain exemplary modified, engineered HA proteins are set forth in Table 15.

[0139] Table 15: Exemplary Modified Influenza HA Proteins

<p>D02a</p> <p>MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILED SHNGKLC LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCTPGYFADYEELRE QLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS YANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD QEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDKCDAKCQTP QGAINSSLPFQNVHPVTIGECPKYVRS AKLRMVTGLRNIPFIQSRGLFGAIAGFIEGGWTG MVDGWYGYHHQNEQSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKL ERRMENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVKNLYEKVKSQ LKNNAK EIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIY STVASSLVLLVSLGAISFWMC SNGLQCRICI</p> <p>(SEQ ID NO: 81)</p>
<p>DO2aRBStrunc00_resG63_G278_graftedontoDO1a</p> <p>MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNLLED SHNGKLC KLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCTPGYFADYEELR EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS YANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD QEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITS DTPVHDCNTTCQTPQ GAINSSLPFQNVHPVTIGECPKYVRS AKLRMATGLRNIPSIQSRGLFGAIAGFIEGGWTGM VDGWYGYHHQNEQSGYAADLKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNKLER RMENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEI GNGCFEFYHKCNNTCMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYST VASSLVLLVSLGAISFWMC SNGLQCRICI</p> <p>(SEQ ID NO: 82)</p>

DO2aRBStrunc00_resG63_G277_graftedontoCal2009

MKAKLLVLLCTFTATYADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGKL
CKLRGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEEL
REQLSSVSSFERFEIFPKESSWPNHVTGTGVSASC SHNGKSSFYRNLLWLTGKNGLYPNLS
KSYANNKEKEVLVLWG VHHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVR
DQEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALSRGAGSGIIISDTPVHDCNTTCQTP
KGAINTSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNIPSIQSRGLFGAIAAGFIEGGWTGM
VDGWYGYHHQNEQSGSYAADLKSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEK
RIENLNKKVDDGFLDIWTYNAELLVLENERLTLDYHDSNVKNLYEKVRSQ LKNNAKEIG
NGCFEFYHKCDNTCMESVKNNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVA
SSLVLVSLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 83)

DO2aRBStrunc00_resG63_G277_graftedontoSC1918

MKAKLLVLLCTFTATYADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SHNGKLC
KLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEELR
EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASC SHNGKSSFYRNLLWLTGKNGLYPNLSKS
YANNKEKEVLVLWG VHHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALSRGSGSGIITS DAPVHDCNTKCQTPH
GAINSSLPFQNIHPVTIGECPKYVRSTKLRLMATGLRNIPSIQSRGLFGAIAAGFIEGGWTGM
DGWYGYHHQNEQSGSYAADQKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFN LERR
IENLNKKVDDGFLDIWTYNAELLVLENERLTDFHDSNVRNLYEKVKSQ LKNNAKEIGN
GCFEFYHKCDDACMESVRNGTYDYPKYSEESKLNREEIDGVKLESMGVYQILAIYSTVA
SSLVLLVSLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 84)

DO2aRBStrunc00_resG63_G277_graftedontoNJ1976

MKAKLLVLLCTFTATYADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLED RHNGKL
CKLGGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEEL
REQLSSVSSFERFEIFPKESSWPNHVTGTGVSASC SHNGKSSFYRNLLWLTGKNGLYPNLS
KSYANNKEKEVLVLWG VHHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVR
DQEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALSRGSGSGIIISDAPVHDCNTKCQTP
KGAINTSLPFQNIHPVTIGECPKYVKSTKLRLMATGLRNIPSIQSRGLFGAIAAGFIEGGWTG
MIDGWYGYHHQNEQSGSYAADQRSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNHLE
KRIENLNKKVDDGFLDIWTYNAELLVLENERLTDFHDSNVKNLYEKVRSQ LRNNAKEI
GNGCFEFYHKCDDTCMESVKNNGTYDYPKYSEESKLNREEIDGVKLESTRIYQILAIYSTV
ASSLVLLVSLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 85)

DO2aRBStrunc01_resV125_G277_graftedontoDO1a

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNLLED SHNGKLC
KLKGIAPLQLGKCSVAGWILGNPECESLSTASSWSYIVETSSPDNGTCYPGYFADYEELR
EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS
YANNKEKEVLVLWGVBHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRIYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSDTPVHDCNTTCQTPQ
GAINSSLFPQNVHPVTIGECPKYVRS AKLRMATGLRNIPSIQSRGLFGAIAGFIEGGWTGM
VDGWYGYHHQNEQGSYAADLKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNKLER
RMENLNKKVDDGFLDIWTYNAELLVLENERLTDFHDSNVKNLYEKVKSQ LKNNAKEI
GNGCFEFYHKCNNTCMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYST
VASSLVLLVSLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 86)

DO2aRBStrunc01_resV125_G277_graftedontoCal2009

MKAKLLVLLCTFTATYADTLCIGYHANNSTDVDTVLEKNVTVTHSVNLLEDKHNGKL
CKLRGVAPLHLGKCNIAGWILGNPECESLSTASSWSYIVETPSSDNGTCYPGDFIDYEELR
EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS
YANNKEKEVLVLWGVBHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRIYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGAGSGIIISDTPVHDCNTTCQTPK
GAINSSLFPQNIHPITIGKCPKYVKSTKLRLATGLRNIPSIQSRGLFGAIAGFIEGGWTGMV
DGWYGYHHQNEQGSYAADLKSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKR
IENLNKKVDDGFLDIWTYNAELLVLENERLTLDYHDSNVKNLYEKVRSQ LKNNAKEIGN
GCFEFYHKCDNTCMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVAS
SLVLVSLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 87)

DO2aRBStrunc01_resV125_G277_graftedontoSC1918

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNLLED SHNGKLC
KLKGIAPLQLGKCNIAGWLLGNPECDLLTASSWSYIVETSNSENGTCYPGDFIDYEELR
EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS
YANNKEKEVLVLWGVBHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRIYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGSGSGIITSDAPVHDCNTKCQTPH
GAINSSLFPQNIHPVTIGECPKYVRSTKLRLMATGLRNIPSIQSRGLFGAIAGFIEGGWTGMI
DGWYGYHHQNEQGSYAADQKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFN NLERR
IENLNKKVDDGFLDIWTYNAELLVLENERLTDFHDSNVRNLYEKVKSQ LKNNAKEIGN
GCFEFYHKCDDACMESVRNGTYDYPKYSEESKLNREEIDGVKLESMGVYQILAIYSTVA
SSLVLLVSLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 88)

DO2aRBStrunc01_resV125_G277_graftedontoNJ1976

MKAKLLVLLCTFTATYADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDHRNGKL
CKLGGIAPLHLGKCNIAGWLLGNPECELLLTVSSWSYIVETSNSDNGTCYPGDFINYEEL
REQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLS
KSYANNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVR
DQEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALS RGS GSGIISDAPVHDCNTKCQTP
KGAINTSLPFQNIHPVTIGECPKYVKSTKL R MATGLRNIPSIQSRGLFGAIAGFIEGGWTG
MIDGWYGYHHQNEQSGSYAADQRSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNHLE
KRIENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVKNLYEKVRSQLRNNAKEI
GNGCFEFYHKDDTCMESVKNGTYDYPKYSEESKLNREEIDGVKLESTRIYQILAIYSTV
ASSLVLLVSLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 89)

DO2aRBStrunc02_resP135_P269_graftedontoDO1A

MKAKLLVLLCTFTATYADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SHNGKLC
KLKGIAPLQLGKCSVAGWILGNPECESLSTASSWSYIVETSSPDNGTCYPGYFADYEELR
EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS
YANNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALS RGF GSGIITS DTPVHDCNTTCQTPQ
GAINSSLPFQNVHPVTIGECPKYVRS AKLR MATGLRNIPSIQSRGLFGAIAGFIEGGWTGM
VDGWYGYHHQNEQSGSYAADLKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNKLER
RMENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEI
GNGCFEFYHKCNNTCMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYST
VASSLVLLVSLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 90)

DO2aRBStrunc02_resP135_P269_graftedontoCal2009

MKAKLLVLLCTFTATYADTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDKHNGKL
CKLRGVAPLHLGKCNIAGWILGNPECESLSTASSWSYIVETPSSDNGTCYPGDFIDYEELR
EQLSSVSSFERFEIFPKESSWPNHVTGTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKS
YANNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRINYWTLLEPGDTIIFEANGNLIAPRYAFAMERNAGSGIISDTPVHDCNTTCQTPK
GAINTSLPFQNIHPITIGKCPKYVKSTKLRLATGLRNIPSIQSRGLFGAIAGFIEGGWTGMV
DGWYGYHHQNEQSGSYAADLKSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKR
IENLNKKVDDGFLDIWTYNAELLV LLENERTLDYHDSNVKNLYEKVRSQ LKNNAKEIGN
GCFEFYHKCDNTCMESVKNGTYDYPKYSEEAKLNREEIDGVKLESTRIYQILAIYSTVAS
SLVLVVSLGAISFWMCSNGSLQCRICI

(SEQ ID NO: 91)

DO2aRBStrunc02_resP135_P269_graftedontoSC1918

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTTHSVNLLED SHNGKLC
KLKGIAPLQLGKCNIA GWLLGNPECDLLLTASSWSYIVETSNSENGTCYPGDFIDYEELR
EQLSSVSSF EKFEIFPKESSWP NHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSK
SYANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRINY YWTLLEPGDTIIFEANGNLIAPWYAFALNRGSGSGIITS DAPVHDCNTKCQTPH
GAINSSLPFQNIHPVTIGEC PKYVRSTKL RMATGLRNIPSIQSRGLFGA IAGFIEGGWTGMI
DGWYGYHHQNEQSGYAADQKSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFN LERR
IENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVRNLYEKVKSQ LKNNAKEIGN
GCFEFYHKCDDACMESVRNGTYDYPKYSEESKLNREEIDGVKLESMGVYQILAIYSTVA
SSLVLLVSLGAISFWMC SNGSLQCRICI

(SEQ ID NO: 92)

DO2ARBStrunc02_resP135_P269_graftedontoNJ1976

MKAKLLVLLCTFTATYADTLCIGYHANNSTDVDTVLEKNVTVTTHSVNLLED RHNGKL
CKLGGIAPLHLGKCNIA GWLLGNPECELLTVSSWSYIVETSNSDNGTCYPGDFINYEEL
REQLSSVSSFERFEIFPKESSWP NHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLS
KSYANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVR
DQEGRINY YWTLLEPGDTIIFEANGNLIAPRYAFAMNRGSGSGIISDAPVHDCNTKCQTP
KGAIN TSLPFQNIHPVTIGEC PKYVKSTKL RMATGLRNIPSIQSRGLFGA IAGFIEGGWTG
MIDGWYGYHHQNEQSGYAADQRSTQNAIDGITNKVNSVIEKMNTQFTAVGKEFNHLE
KRIENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVKNLYEKVRSQ LRNNAKEI
GNGCFEFYHKCDDTCMESVKNGTYDYPKYSEESKLNREEIDGVKLESTRIYQILAIYSTV
ASSLVLLVSLGAISFWMC SNGSLQCRICI

(SEQ ID NO: 93)

SMART_NC_DO2a_NGlyMod

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTTHSVNILED SHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCPYGYFADYEELRE
QLSSVSSFERFEIFPKESSWP NHDSN-GVSASCSHNGKSSFYRNLLWLTGKNG
LYPKLSKSYANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIA
KRPKVRDQEGRINY YWTLLEPGDTIIFEANGNLIAPWYAFALS RGFSGSIITSNAPMDKC
DAKCQTPQGAINSSLPFQNVHPVTIGEC PKYVRS AKLRMVTGLRNIPFIQSRGLFGA IAGF
IEGGWTGMVDGWYGYHHQNEQSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAV
GKEFNKLERRMENLNKKVDDGFLDIWTYNAELLV LLENERTLDFHDSNVKNLYEKVKS
QLKNNAKEIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMG
VYQILAIYSTVASSLVLLVSLGAISFWMC SNGSLQCRICI

(SEQ ID NO: 94)

SMART_NC_DO2a_NGlyMod+loopInsertion(CA09)

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILEDSHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEELRE
QLSSVSSFERFEIFPKESSWPNHDSNKGVSASCSHNGKSSFYRNLLWLTGKNGLYPKLSK
SYANNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDKCDACQTP
QGAINSSLPFQNVHPVTIGECPKYVRS AKLRMVTGLRNIPFIQSRGLFGAIAGFIEGGWTG
MVDGWYGYHHQNEQGSYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKL
ERRMENLNKKVDDGFLDIWTYNAELLVLENERTLDFHDSNVKNLYEKVKSQKNNNAK
EIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIY
STVASSLVLLVSLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 95)

SMART_NC_DO2A_NGLYMOD+LOOPINSERTION(SC18)

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILEDSHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEELRE
QLSSVSSFERFEIFPKESSWPNHETTKGVSASCSHNGKSSFYRNLLWLTGKNGLYPKLSKS
YANNKEKEVLVLWGVHPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRD
QEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDKCDACQTP
QGAINSSLPFQNVHPVTIGECPKYVRS AKLRMVTGLRNIPFIQSRGLFGAIAGFIEGGWTG
MVDGWYGYHHQNEQGSYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKL
ERRMENLNKKVDDGFLDIWTYNAELLVLENERTLDFHDSNVKNLYEKVKSQKNNNAK
EIGNGCFEFYHKCNDECMESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIY
STVASSLVLLVSLGAISFWMCNGLSLQCRICI

(SEQ ID NO: 96)

SMART_NC_DO2a_mods_outside_ch65_eptiope1

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILEDSHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEELRE
QLSSVSSFERFEIFPKTSSWPNHVT-
GVSASCPHAGAKSFYRNLLWLTGKNGLYPNLSKSYANNKEKEVLVLWGVHPPNIGDQ
RALYQNADAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGDTIIFEATGNLI
APWYAFALSRGFGSGIITSNAPMDKCDACQTPQGAINSSLPFQNVHPVTIGECPKYVRS
AKLRMVTGLRNIPFIQSRGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQGSYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELL
VLENERTLDFHDSNVKNLYEKVKSQKNNNAKEIGNGCFEFYHKCNDECMESVKNGTY
DYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMCNGLSLQ
CRICI

(SEQ ID NO: 97)

SMART_NC_DO2a_mods_outside_ch65_eptiope2

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNILEDSHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCTPGYFADYEELRE
QLSSVSSFERFEIFPKESSWPNHVT-
GVSASCPHAGAKSFYRNLLWLTGKNGLYPNLSKSYANNKEKEVLVLWGVHHPNIGDQ
RALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYWTLLEPGDTIIFEANGNLI
APWYAFALSRGFGSGIITSNAPMDKCDKACQTPQGAINSSLPFQNVHPVTIGECPKYVRS
AKLRMVTGLRNIPFIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQGSYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELL
VLLNERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCNDECMESVKNGTY
DYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMCNGLSQ
CRICI

(SEQ ID NO: 98)

SMART_NC_DO2a_mods_outside_ch65_eptiope3

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNILEDSHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCTPGYFADYEELRE
QLSSVSSFERFEIFPKESSWPNHVT-
GVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYANNKEKEVLVLWGVHHPNIGDQ
RALYQNADAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYWTLLEPGDTIIFEANGNL
IAPWYAFALSRGFGSGIITSNAPMDKCDKACQTPQGAINSSLPFQNVHPVTIGECPKYVRS
AKLRMVTGLRNIPFIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQGSYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELL
VLLNERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCNDECMESVKNGTY
DYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMCNGLSQ
CRICI

(SEQ ID NO: 99)

SMART_NC_DO2a_mods_outside_ch65_eptiope1-noGly

MKAKLLVLLCTFTATYADTICIGYHANNSTDVDTVLEKNVTVTHSVNILEDSHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCTPGYFADYEELRE
QLSSVSSFERFEIFPKTSSWPNHNTT-
GVSASCPHAGAKSFYRNLLWLTGKNGLYPKLSKSYANNKEKEVLVLWGVHHPNIGDQ
RALYQNADAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYWTLLEPGDTIIFEATGNLI
APWYAFALSRGFGSGIITSNAPMDKCDKACQTPQGAINSSLPFQNVHPVTIGECPKYVRS
AKLRMVTGLRNIPFIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQNEQGSYAADQKS
TQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWTYNAELL
VLLNERTLDFHDSNVKNLYEKVKSQKNNAKEIGNGCFEFYHKCNDECMESVKNGTY
DYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSLVLLVSLGAISFWMCNGLSQ
CRICI

(SEQ ID NO: 100)

SMART_NC_DO2a_mods_outside_ch65_eptiope2-noGly

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILED SHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEELRE
QLSSVSSFERFEIFPKESSWP NHNTT-GVSASCPHAGAKSFYRNLLWLTGKNG
LYPKLSKSYANNKEKEVLVLWGVHHPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIA
KRPKVRDQEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDKC
DAKCQTPQGAINSSLPFQNVHPVTIGEC PKYVRS AKLRMVTGLRNIPFIQSRGLFGAIAGF
IEGGWTGMVDGWYGYHHQNEQSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAV
GKEFNKLERRMENLNKKVDDGFLDIWYTNAELLV LLENERTLDFHDSNVKNLYEKVKS
QLKNNAKEIGNGC FEFYHKCNDECMESVKN GTYDYPKYSEESKLNREKIDGVKLESMG
VYQILAIYSTVASSLVLLVSLGAISFWMC SNGLQCRICI

(SEQ ID NO: 101)**SMART_NC_DO2a_mods_outside_ch65_eptiope3-noGly**

MKAKLLVLLCTFTATYADTICIGYHANNSTDTVDTVLEKNVTVTTHSVNILED SHNGKLC
LLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVEKPNPENGTCYPGYFADYEELRE
QLSSVSSFERFEIFPKESSWP NHNTT-GVSASCSHNGKSSFYRNLLWLTGKNG
LYPKLSKSYANNKEKEVLVLWGVHHPNIGDQRALYQNADAYVSVVSSHYSRRFTPEIA
KRPKVRDQEGRINYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDKC
DAKCQTPQGAINSSLPFQNVHPVTIGEC PKYVRS AKLRMVTGLRNIPFIQSRGLFGAIAGF
IEGGWTGMVDGWYGYHHQNEQSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAV
GKEFNKLERRMENLNKKVDDGFLDIWYTNAELLV LLENERTLDFHDSNVKNLYEKVKS
QLKNNAKEIGNGC FEFYHKCNDECMESVKN GTYDYPKYSEESKLNREKIDGVKLESMG
VYQILAIYSTVASSLVLLVSLGAISFWMC SNGLQCRICI

(SEQ ID NO: 102)

[0140] In various embodiments, engineered HA mosaic polypeptides as described herein comprise combinations of epitope patterns on a particular viral backbone sequence. Multiple epitopes can be assembled on to any viral backbone as desired. Exemplary viral backbone sequences include A/New Caledonia/20/1999, A/California/07/2009, and a consensus (e.g., 1918-2011) sequence. In some embodiments, engineered HA mosaic polypeptides as described herein comprise a New Caledonia 99 or California 09 backbone sequence.

[0141] In some embodiments, an engineered HA polypeptide has a sequence at least about 90% (e.g., at least about 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%) identical to a sequence that appears in Table 13, 14, or 15. In some embodiments, an engineered HA polypeptide has a sequence that is substantially identical to a sequence that appears in Table 13, 14, or 15. In some embodiments, an engineered HA polypeptide has a sequence that is identical to a sequence that appears in Table 13, 14, or 15.

Expression of Engineered Structural Influenza Proteins

[0142] Optimized nucleotide sequences obtained by the methods described herein may be expressed in a cell-free system or in a host cell using known methods. Expression of optimized nucleotide sequences of the present invention may be regulated by a second nucleic acid sequence so that the molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the optimized nucleotide sequence of the invention may be controlled by a promoter and/or enhancer element, which are known in the art.

[0143] Nucleic acid constructs of the present invention are inserted into an expression vector or viral vector by methods known to the art, and nucleic acid molecules are operatively linked to an expression control sequence.

[0144] An expression vector containing a nucleic acid molecule is transformed into a suitable host cell to allow for production of the protein encoded by the nucleic acid constructs. Exemplary host cells include prokaryotes (e.g., *E. coli*) and eukaryotes (e.g., a COS, 293 or CHO cell). Host cells transformed with an expression vector are grown under conditions permitting production of an engineered structural influenza protein followed by recovery of the engineered protein.

[0145] Vectors comprising the nucleic acid molecules encoding recombinant structural influenza proteins are also provided. The vector can be any suitable vector for expression of the engineered structural influenza protein, such as a mammalian expression vector. In particular examples, the vector is the pTR600 expression vector (U.S. Patent Application Publication No. 2002/0106798, herein incorporated by reference; Ross et al., *Nat Immunol.* 1(2): 102-103, 2000; Green et al., *Vaccine* 20:242-248, 2001). In some examples, the vector includes a promoter operably linked to the optimized nucleotide sequence encoding the engineered structural influenza protein. In particular examples, the promoter is a CMV promoter.

[0146] Engineered structural influenza polypeptides may be purified by any technique known in the art. For example, not wishing to be bound by theory, engineered structural influenza polypeptides may be recovered from cells either as soluble polypeptides or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify engineered structural influenza polypeptides, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used. Engineered structural influenza polypeptides of

the present invention may also be recovered from conditioned media following secretion from eukaryotic or prokaryotic cells.

Reverse Genetics Methods

[0147] The optimized nucleotide sequences obtained by the methods described herein can be combined with one or more donor viruses and used in a reverse genetics system to produce an infectious reassortant influenza virus. As discussed above, reverse genetics systems can be used produce infectious, reassortant viruses, or attenuated viruses from their cDNAs. The reverse genetics methods are well-known by the one skilled in the art and include, but are not limited to, the methods using the plasmids described in Neuman et al, 1999, Proc Natl Acad Sci USA, 96(16):9345-9350; Neumann et al, 2005, Proc Natl Acad Sci USA, 102(46):16825-16829; Zhang et al, 2009, J Virol, 83(18):9296-9303; Massin et al, 2005, J Virol, 79(21):1381 1 -13816; Murakami et al, 2008, 82(3):1605-1609; and/or the cells described in Neuman et al, 1999, Proc Natl Acad Sci USA, 96(16):9345-9350; Neumann et al, 2005, Proc Natl Acad Sci USA, 102(46): 16825- 16829; Zhang et al, 2009, J Virol, 83(18):9296-9303; Massin et al, 2005, J Virol, 79(21):1381 1 -13816; Murakami et al, 2008, 82(3):1605-1609; Koudstaal et al, 2009, Vaccine, 27(19):2588-2593; Schickli et al, 2001 , Philos Trans R Soc Lond Biol Sci, 356(1416):1965-1973; Nicolson et al, 2005, Vaccine, 23(22):2943-2952; Legastelois et al, 2007, Influenza Other Respi Viruses, 1 (3):95-104; Whiteley et al, 2007, Influenza Other Respi Viruses, 1 (4): 157-166.

[0148] In certain embodiments, the reverse genetics method may be:

[0149] (i) the 16 plasmid method, such as the method described by Neuman et al, 1999, Proc Natl Acad Sci USA, 96(16):9345-9350, and in US 2009/0246830 or US 2011/0143424 (each of which is hereby incorporated by reference in its entirety), in which the influenza virus is produced by transfecting cells, using a polyamine derivative (Trans IT-LT1), with 8 plasmids each containing a cDNA complementary to one influenza vRNA under the control of an RNA polymerase I promoter and an RNA polymerase I terminator, and 8 plasmids each containing a cDNA complementary to one of the PA, PB1 , PB2, NP, HA, NA, M and NS mRNAs under the control of RNA polymerase II promoter. In particular, the cells are human kidney embryonic adherent cells (293T cell line);

[0150] (ii) the 12 plasmid method, such as the method described by Fodor et al, 1999, J Virol, 73(1 1):9679-9682, and in US 2004/0142003, US 2012/0058538 (each of which is hereby

incorporated by reference in its entirety) in which the influenza virus is produced by transfecting a first cell type with 8 plasmids each containing a cDNA complementary to one influenza vRNA under the control of an RNA polymerase I promoter and an RNA polymerase I terminator (hepatitis delta ribozyme), and 4 plasmids each containing a cDNA complementary to one of the NP, PA, PB1 and PB2 mRNAs under the control of RNA polymerase II promoter, and by further amplifying the virus on a second cell type. In particular, said first cell type is Vero cells and said second cell type is MDBK;

[0151] (iii) the 13 plasmid method, such as the method described by De Wit et al, 2007, Journal of General Virology, 88:1281 -1287 (which is hereby incorporated by reference in its entirety) in which the influenza virus is produced by transfecting cells with 8 plasmids each containing a cDNA complementary to one influenza vRNA under the control of an T7 RNA polymerase promoter and an T7 RNA polymerase terminator, 4 plasmids each containing a cDNA complementary to one of the NP, PA, PB1 and PB2 mRNAs under the control of RNA polymerase II, and one plasmid containing the cDNA complementary to the mRNA encoding the T7 RNA polymerase and a nuclear localization signal under the control of RNA polymerase II. In particular, the transfected cells are Vero, 293T, or QT6 (fibrosarcoma cell line from Japanese quail) cells.

[0152] (iv) the 8 plasmid method, such as the method described by Hoffmann et al, 2000, PNAS, 97(11):6108-6113 and in WO 01/83794 (each of which is hereby incorporated by reference in its entirety) in which each plasmid is capable of expressing both mRNA and vRNA(s). Thus each plasmid contains cDNA complementary to one influenza vRNA and two transcription cassettes instead of one as in the preceding case. The cDNA complementary of each of the eight influenza virus vRNAs is inserted between the polymerase I terminator and the polymerase I promoter. This polymerase I transcription unit is flanked by the polymerase II promoter and a polyadenylation signal. The first transcription cassette allows the transcription of cDNA in the form of a vRNA. The second transcription cassette allows the transcription of cDNA in the form of mRNA which is then translated into viral protein(s) using the cellular machinery. With the aid of this double cassette system for transcription, also called Pol I-Pol II system, the cDNA of the same plasmid is transcribed both in the form of vRNA and in the form of mRNA. This manifests itself at the level of the transfected cell by the expression of a vRNA and of one or more viral proteins. In particular, a co-culture of adherent MDCK cells and of 293T cells and, as transfection agent, a polyamine derivative (Trans IT-LT1) are used.

[0153] (v) the 3 plasmid method, such as the method described by Neumann et al, 2005, PNAS, 102(46): 16825-16829 (which is hereby incorporated by reference in its entirety), in which the influenza virus is produced by transfecting cells with one plasmid containing the 8 cDNAs complementary to PB2, PB1, PA, HA, NP, NA, M and NS vRNAs each under the control of an RNA polymerase I promoter and a polymerase I terminator and 2 plasmids, the first one containing the 3 cDNA complementary to one of the PB2, PB1 and PA mRNAs and the second one containing the cDNA complementary to the NP mRNA, under the control of a RNA polymerase II promoter. In particular, the transfected cells are 293T or Vero.

[0154] (vi) the 1 plasmid method, such as the method described by Zhang et al, J.Virol., 83(18): 9296-9303 (which is hereby incorporated by reference in its entirety), in which the influenza virus is produced by transfecting cells with one plasmid containing the 8 cDNAs complementary to PB2, PB1, PA, HA, NP, NA, M and NS vRNA under the control of murine polymerase I terminator and a chicken RNA polymerase I promoter and with a polymerase II promoter and a polyadenylation signal between PB2, PB1, PA and NP cDNAs. In particular, the transfected cells are CEF cells.

[0155] (vii) the method described in WO 2005/062820 (which is hereby incorporated by reference in its entirety) using two different cellular systems: in a first step, cells are transfected with 8 bidirectional plasmids with the Poll-PollI system (Pol/Poll) and then in a second step, the transfected cells are cultured with cells from another cell line that is very permissive for the influenza virus in order to amplify the production of the influenza virus. In particular, said transfected cells in the first step are Vero cells, and said other cell line in the second step are CEK or CEF cell lines which are lines derived from chicken embryo cells.

[0156] Thus, certain embodiments are directed to a method of producing an infectious reassortant influenza virus ("reverse genetics" method), the method comprising transfecting cells with an expression vector comprising an optimized nucleotide sequence encoding a structural influenza protein and one or more donor vectors, and producing the infectious reassortant influenza virus (or seed virus). In certain embodiments, the cells are mammalian cells, including, but not limited to, Vero cells, HEK-293 cells, MDCK cells, or Chinese Hamster Ovary (CHO) cells and combinations thereof. In some embodiments, the methods described herein and the optimized nucleotide sequences thereof are used with the vectors, recombination cassettes and overall system described in WO2014/019990 and U.S. Application No. 14/419,235, (U.S. Publication No. 2015-0191703 A1), which are incorporated herein by reference in their entirety.

[0157] The supernatant of the transfected cells contains infectious reassortant influenza virus, which can be harvested and/or isolated and used as an infectious seed virus to infect a separate population of cells or eggs. Alternatively, after the transfection step, cells or eggs can be added *in situ* to the transfected cells to allow the proliferation of infectious influenza virus. In certain embodiments, the cells are mammalian cells, including, but not limited to, Vero cells or Chinese Hamster Ovary (CHO) cells.

[0158] It is well understood that the infection of cells with the seed virus is made under culture conditions well known by the skilled in the art that allow the proliferation of infectious influenza virus. The proliferation of the infectious influenza virus can be further amplified by successive infections of the cell populations or any other highly permissive cell populations, or by infecting the allantoic cavity of embryonated hen's eggs.

[0159] The transfected mammalian cells are preferably adapted for culture in serum-free medium and/or animal component free conditions. Cell adaptation to culture in serum free medium may readily achieved by the one skilled in the art by progressively passaging cells on media containing decreasing serum amounts, until the cells can successfully survive and proliferate in a serum-free medium.

[0160] Cells can be transfected by any method known by the one skilled in the art. For example, transfection may be performed by membrane electroporation, nuclear electroporation. In certain embodiments, transfection is performed by nuclear electroporation. The expression "nuclear electroporation" is understood to mean a method of transfection of nucleic acids by means of one or more electric shocks whose intensity is sufficient to increase the number of nuclear pores and/or the permeability thereof.

[0161] In certain embodiments, the recombinant virus comprises an HA influenza polypeptide encoded by an optimized nucleotide sequence as described herein, a wild-type NA polypeptide from an influenza strain and a backbone of internal protein genes from a donor virus (e.g., influenza A/Puerto Rico/8/34 (PR8)) that confers a high yield in eggs. For example, six plasmids encoding the internal proteins of the high-growth influenza A/Puerto Rico/8/34 (PR8) donor virus can be co-transfected with a plasmid encoding an engineered influenza structural polypeptide as described herein and a wild-type neuraminidase (NA) glycoprotein into qualified mammalian cells (e.g., Vero cells), followed by isolation of the recombinant virus. Recombinant viruses containing internal protein genes from the PR8 virus may be used to prepare inactivated influenza virus vaccines (see, e.g., Fodor, E. et al. *Rescue of influenza A*

virus from Recombinant DNA. J. Virol., 1999, 73, 9679-9682; incorporated by reference herein).

Influenza virus-like particles (VLPs)

[0162] In some embodiments, the present invention provides for influenza virus-like particles (VLPs) and combinations thereof comprising one or more of the engineered structural influenza proteins encoded by an optimized nucleotide sequence as described herein. The influenza VLPs are, in some embodiments, generally made up of HA, NA and virus structural (e.g., HIV gag) proteins. Production of influenza VLPs is known in the art and will be readily apparent to persons of skill upon reading the present disclosure. For example, influenza VLPs may be produced by transfection of host cells with plasmids encoding the HA, NA and HIV gag proteins. To give but one example, a suitable host cell includes a human cell (e.g., HEK293T). After incubation of the transfected cells for an appropriate time to allow for protein expression (such as for approximately 72 hours), VLPs may be isolated from cell culture supernatants. In some embodiments, influenza VLPs as disclosed herein may be used as influenza vaccines to elicit a broadly neutralizing immune response against influenza viruses.

Whole Influenza Viruses

[0163] Also provided are whole recombinant influenza viruses comprising one or more of the engineered influenza structural proteins described herein. The recombinant influenza viruses can be produced by plasmid-based reverse genetics, as described herein, and cell-based or egg-based technologies. Recombinant viruses containing internal protein genes from a donor virus may be used to prepare inactivated influenza virus vaccines (see, e.g., Fodor, E. et al. *Rescue of influenza A virus from Recombinant DNA*. J. Virol., 1999, 73, 9679-9682; incorporated by reference herein). Distinct recombinant influenza viruses, each comprising a different recombinant, structural influenza polypeptide, can also be separately produced and then combined into combinations/cocktails. The recombinant influenza virus combinations/cocktails can be used as influenza vaccines to elicit a protective immune response against influenza viruses; for example, they can be administered as components of a live-attenuated or split-inactivated vaccine.

[0164] Thus, in some embodiments, the present invention provides inactivated influenza vaccines comprising a structural influenza polypeptide (or combinations or cocktails thereof)

encoded by an optimized nucleotide sequence, wherein the vaccines comprise one of three types of antigen preparation: inactivated whole virus, sub-virions where purified virus particles are disrupted with detergents or other reagents to solubilize the lipid envelope (“split” vaccine) or purified structural influenza polypeptide (“subunit” vaccine). In some embodiments, virus can be inactivated by treatment with formaldehyde, beta-propiolactone, ether, ether with detergent (such as TWEEN-80[®]), cetyl trimethyl ammonium bromide (CTAB) and Triton N101, sodium deoxycholate and tri(n-butyl) phosphate. Inactivation can occur after or prior to clarification of allantoic fluid (from virus produced in eggs); the virions are isolated and purified by centrifugation (Nicholson et al., eds., 1998, *Textbook of Influenza*, Blackwell Science, Malden, MA; incorporated herein by reference). To assess the potency of the vaccine, the single radial immunodiffusion (SRD) test can be used (Schild et al., 1975, *Bull. World Health Organ.*, 52:43-50 & 223-31; Mostow et al., 1975, *J. Clin. Microbiol.*, 2:531; both of which are incorporated herein by reference).

[0165] In some embodiments, influenza virus for use in vaccines is grown in eggs, for example, in embryonated hen eggs, in which case the harvested material is allantoic fluid. Alternatively or additionally, influenza virus or an influenza structural polypeptide encoded by an optimized nucleotide sequence may be produced from any method using tissue culture to grow the virus. Suitable cell substrates for growing the virus or otherwise recombinantly producing the engineered, structural influenza polypeptides include, for example, CHO cells, dog kidney cells such as MDCK or cells from a clone of MDCK, MDCK-like cells, monkey kidney cells such as AGMK cells including Vero cells, cultured epithelial cells as continuous cell lines, 293T cells, BK-21 cells, CV-1 cells, or any other mammalian cell type suitable for the production of influenza virus (including upper airway epithelial cells) for vaccine purposes, readily available from commercial sources (*e.g.*, ATCC, Rockville, Md.). Suitable cell substrates also include human cells such as MRC-5 cells. Suitable cell substrates are not limited to cell lines; for example primary cells such as chicken embryo fibroblasts are also included.

Methods for Preparing Pharmaceutical Compositions

[0166] Also provided herein are methods for preparing an influenza vaccine composition, the method comprising:

a) generating a seed virus by transfecting mammalian cells with a set of expression vectors, one or more of which comprises an optimized nucleotide sequence encoding an engineered influenza structural protein;

- b) harvesting the seed virus;
- c) infecting eggs or mammalian cells with the seed virus to produce an infectious influenza virus;
- d) harvesting the infectious influenza virus after multiplication in the eggs of mammalian cells;
- e) purifying the harvested infectious influenza virus,
- d) optionally inactivating the purified virus, and
- e) mixing the purified virus with a pharmaceutically acceptable carrier.

[0167] In some embodiments, the expression vectors are those described in WO2014/019990 and U.S. Application No. 14/419,235, (U.S. Publication No. 2015-0191703 A1), which are incorporated herein by reference in their entirety; in some embodiments, the vectors comprise the recombination cassettes described in WO2014/019990.

[0168] In some embodiments, said set of expression vectors comprises: expression vectors allowing the expression of mRNAs encoding at least influenza PB1 , PB2, PA and NP proteins, and expression vectors allowing the expression of at least influenza PB1 , PB2, PA, NP, M, NS, HA and NA vRNAs, or the corresponding cRNAs. Expression of said set of expression vectors allows (i) the formation of the ribonucleoprotein complex (RNP) containing the influenza vRNA(s), and (ii) the generation of infectious influenza viruses in said transfected cells. In particular embodiments, the expression vectors allowing the expression of mRNAs encoding influenza PB1 , PB2, PA and NP proteins comprise four different uni-directional plasmids, each plasmid containing a cDNA complementary to a mRNA encoding one of the four distinct proteins selected from PB1 , PB2, PA and NP influenza proteins under the control of a promoter that binds to RNA polymerase II, and the expression vectors allowing the expression of influenza PB1 , PB2, PA, NP, M, NS, HA and NA vRNAs, or the corresponding cRNAs, comprise eight different uni-directional plasmids, each plasmid containing a cDNA complementary to one of the eight distinct vRNAs selected from said PB1 , PB2, PA, NP, M, NS, HA and NA influenza vRNAs, or to the corresponding cRNAs, under the control of a promoter that binds to RNA polymerase I. In some embodiments, each plasmid containing a cDNA complementary to one of said influenza PB1 , PB2, PA, NP, M, NS, HA and NA vRNAs (e.g., a cDNA comprising an optimized nucleotide sequence as described herein), or the corresponding cRNAs, under the control of a promoter that binds to RNA polymerase I has been obtained by cloning said cDNA sequence into a vector comprising, in the 5' to 3' sense :

a) a promoter that binds to RNA polymerase I, or a T7 RNA polymerase; b) a recombination cassette comprising, in the 5' to 3' sense:

- an inverted complementary recognition sequence for a first restriction enzyme which has its cutting site outside of its recognition sequence and produces sticky ends;
- a restriction site for a second restriction enzyme which has its cutting site inside of its recognition sequence;
- a restriction site for a third restriction enzyme which has its cutting site inside of its recognition sequence; and
- a recognition sequence for said first restriction enzyme which has its cutting site outside of its recognition sequence and produces sticky ends; wherein said second and third restriction enzymes are different; and

c) a terminator sequence. In particular embodiments, when the promoter binds to RNA polymerase I, said terminator sequence is hepatitis delta ribozyme sequence, and when the promoter binds to T7 RNA polymerase, said terminator sequence is T7 polymerase terminator sequence.

[0169] The purification may be brief and may be limited to a step of concentrating the virus by centrifugation after having generally clarified the harvested infectious virus. The purification may be supplemented with centrifugation step carried out for example by means of sucrose density gradients (EP 0 7760362). Chromatographic methods may also be carried out in order to purify the virus. A suspension of purified whole viruses is thus obtained which can be further processed to get the final vaccine composition. The purified virus suspension may also undergo subsequent treatments. Flu virus-derived products are thus obtained. The viral suspension may be fragmented using detergents or lipid solvents according to methods well known to those skilled in the art, in order to manufacture, for example, vaccines based on fragmented or split viruses, virosomes, or subunit vaccines containing the engineered influenza virus structural protein. The fragmented or split viruses, the virosomes containing the engineered influenza structural protein and the subunit vaccines containing the engineered influenza structural protein which are obtained from the purified virus are considered to be flu virus-derived products.

[0170] The final vaccine composition can be made up of whole inactivated influenza virus or attenuated influenza virus. The inactivation of the viral suspension is carried out by conventional means, using β -propiolactone (E. Budowsky et al. 1991, Vaccine, 9: 319-325;

1991, *Vaccine*, 9: 398-402; 1993, *Vaccine*, 11 : 343-348), ethyleneimine or derivatives (D. King 1991, *Avian Dis.* 35: 505-514) or formol (EP 0 776 0362). The inactivation of the virus can be carried out before or after the purification step.

[0171] The final vaccine composition is generally formulated with a pharmaceutically acceptable carrier. The vaccine composition may also comprise one or more adjuvants. For example, alum, aluminum salts (Baylor et al., 2002, *Vaccine*, 20:S18; incorporated herein by reference) and monophosphoryl lipid A (MPL; Ribí et al., 1986, *Immunology and Immunopharmacology of Bacterial Endotoxins*, Plenum Publ. Corp., NY, p.407; incorporated herein by reference) can be used as adjuvants in human vaccines. Alternatively or additionally, new compounds are currently being tested as adjuvants in human vaccines, such as MF59 (See, e.g., Ott et al., “MF59—Design and Evaluation of a Safe and Potent Adjuvant for Human Vaccines” in *Vaccine Design: The Subunit and Adjuvant Approach* (Powell, M. F. and Newman, M. J. eds.) Plenum Press, New York, 1995, pp. 277-296; incorporated herein by reference); CpG oligodeoxynucleotide (ODN) adjuvants such as CPG 7909 (Cooper et al., 2004, *Vaccine*, 22:3136; incorporated herein by reference); Monophosphoryl lipid A (MPL) adjuvants and lipid A mimetics including AS04 (Didierlaurent, A.M. et al, *J. Immunol.*, 2009, 183: 6186-6197; incorporated by reference herein), monophosphoryl lipid A (MPL, GSK) and glucopyranosyl lipid A GLA (Immune Design Corporation, IDC); AF03 (Klucker, M.F. et al, *J. Pharm Sci.*, 2012, 101: 4490-4500; incorporated herein by reference); the TLR-3 ligand polyinosinic:polycytidylic acid [poly(I:C)]; TLR9 adjuvants such as IC31 (Riedl, K. et al., *Vaccine*, 2008, 26: 3461-3468; incorporated herein by reference); imidazoquinolines (double cyclic organic molecules that act as TLR-7/8 agonists) such as imiquimod (R837) or resiquimod (R848); saponins such as QS21 (Ghochikyan et al., 2006, *Vaccine*, 24:2275; incorporated herein by reference), ISCOMATRIX adjuvant (Duewell, P., et al., *J. Immunol*, 2011, 187: 55-63; incorporated herein by reference), and Matrix-M™ (Novavax).

[0172] Additionally, some adjuvants are known in the art to enhance the immunogenicity of influenza vaccines, such as poly[di(carboxylatophenoxy)phosphazene] (PCCP; Payne et al., 1998, *Vaccine*, 16:92; incorporated herein by reference), interferon- γ (Cao et al., 1992, *Vaccine*, 10:238; incorporated herein by reference), block copolymer P1205 (CRL1005; Katz et al., 2000, *Vaccine*, 18:2177; incorporated herein by reference), interleukin-2 (IL-2; Mbuike et al., 1990, *Vaccine*, 8:347; incorporated herein by reference), and polymethyl methacrylate (PMMA; Kreuter et al., 1981, *J. Pharm. Sci.*, 70:367; incorporated herein by reference).

[0173] The present invention will be more fully understood by reference to the following Examples. All literature citations are incorporated by reference.

EXAMPLES

[0174] *Example 1 – Nucleotide Sequence Optimization of P1, X6, and X1 COBRAs*

[0175] Methods of generating an optimized nucleotide sequence encoding an engineered influenza structural protein were implemented using the P1 (SEQ ID NO: 4), X6 (SEQ ID NO: 5), and X1 (SEQ ID NO: 7) COBRAs. Without optimizing the nucleotide sequences encoding the COBRAs, little to no viral rescue was possible in a reverse genetics system. For each COBRA, two optimized nucleotide sequences were produced: one that was obtained following steps 1-4 in Figure 1 and one that was obtained following steps 1-5 in Figure 1.

[0176] More specifically, for each of the P1, X6, and X1 COBRAs, an optimized nucleotide sequence was obtained by reverse translating the COBRA amino acid sequence, comparing the reverse translated nucleotide sequence to a database of influenza sequences, and optimizing the reverse translated nucleotide sequence according to the rules set forth in Steps 3a and 3b of Figure 1. The optimized nucleotide sequences were also modified by adding the 5' and 3' non-coding regions from the high-titer rescued strain A/PuertoRico/8/34 ("PR8"). These optimized nucleotide sequences are referred to as "codon bias" in Figure 5.

[0177] In the case of PR8, the following 5'- and 3'-terminal nucleotide sequences were used:

[0178] PR8 5' terminal sequence

[0179] AGCAAAAGCAGGGGAAAATAAAAACAACCAAAATGAAGGCAAACCTACT
GGTCCTGTTATGTGCACTTGCAGCTGCAGATGCA (SEQ ID NO: 23)

[0180] PR8 3' terminal sequence

[0181] CAGATTCTGGCGATCTACTCAACTGTCGCCAGTTCCTGGTGCTTTTGGTC
TCCCTGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGATCTTTGCAGTGCAGAA
TATGCATCTGAGATTAGAATTTCAGAGATATGAGGAAAAACACCCTTGTTTCT
(SEQ ID NO: 24)

[0182] The "codon bias" optimized sequences were also further modified by exchanging certain coding regions with other influenza HA proteins. The optimized X6 COBRA sequence was further modified by exchanging the signal peptide at the 5' terminus with a signal peptide from either the COBRA X3 sequence (see Table 12) or a wild type influenza virus

(A/Wellington/24/2000). More specifically, the sequence encoding the signal peptide of the X6 COBRA was exchanged with the following nucleotide sequences encoding the signal peptide from the A/Wellington/24/2000 strain (SEQ ID NO: 25) or the X3 COBRA (SEQ ID NO: 26). The coding sequences are italicized.

[0183] 5' A/Wellington/24/2000 terminal sequence:

ATGAAAGTAAACTACTGGTCCTGTTATGTACATTACAGCTACATATGC (SEQ ID NO: 25)

[0184] 5' X3 COBRA terminal sequence:

*ATGGAAGCAAGACTACTAGTCCTGTTATGTGCATTGTCAGCTACAAATGCAGACACAAT
ATGTATAGGCTACCATGCG* (SEQ ID NO: 26)

[0185] The optimized X1 COBRA sequence was further modified by swapping 5' and 3' termini with the 5' and 3' termini of COBRA A1 or PR8. More specifically, the 5' nucleotide sequence encoding the signal peptide and into an initial part of the ectodomain were swapped with the corresponding COBRA A1 sequence. This exchange introduced changes in the signal peptide but not the ectodomain region (i.e., only codon changes were made in the ectodomain). The 3' terminal region, encoding the transmembrane domain and cytoplasmic tail, was also swapped with the corresponding sequence from COBRA A1. The 5' and 3' COBRA A1 terminal sequences that were exchanged correspond to SEQ ID NO: 27 and SEQ ID NO: 28, respectively. The coding sequences are italicized.

[0186] 5' COBRA A1 terminal sequence:

*ATGAAAGCAAACTACTAGTTCTGTTATGTGCATTACAGCTACATATGCAGACACAATA
TGTATAGGCTACCATGCGAACAACCTCAACCGACACTGTTGACACAGTACTTGAAAAGAA
CGTGACAGTGACACACTCTGTCAACCTACTTGAGGACAGTCACAACGGAAAACCTATGT
CGACTAAAAGGAATAGCCCCACTACAATTGGGT* (SEQ ID NO: 27)

[0187] 3' COBRA A1 terminal sequence:

*AAGAACAATGCCAAAGAAATAGGAAACGGGTGTTTTGAATTCTACCACAAGTGTAACAA
TGAATGCATGGAAAGTGTGAAAAATGGAACCTATGACTATCCAAAATATTCCGAGGAAT
CAAAGTTAAACAGGGAAAAAATTGATGGAGTGAAATTGGAATCAATGGGAGTCTATCAG
ATTCTGGCGATCTACTCAACTGTCGCCAGTTCAGTGGTGCTTTGGTCTCCCTGGGGG
CAATCAGCTTCTGGATGTGTTCTAATGGGTCTTGCAGTGTAGAATATGCATCTGAGAT
TAGAATTTGAGAGATATGAGGAAAAACACCCTTGTTTCT* (SEQ ID NO: 28)

[0188] The 5' nucleotide sequence encoding the signal peptide (but not including any portion of the ectodomain) of COBRA X1 was also swapped with the corresponding PR8 sequence.

This exchange did not introduce any change in the amino acid sequence. The 3' terminal region of PR8, encoding the transmembrane domain and cytoplasmic tail, was also swapped with the corresponding 3' sequence from COBRA X1. The 5' and 3' PR8 terminal sequences that were exchanged correspond to SEQ ID NO: 29 and SEQ ID NO: 30, respectively. The coding sequences are italicized.

[0189] 5' PR8 terminal sequence:

*AGCAAAAGCAGGGGAAAATAAAAACAACCAAAATGAAGGCAAACCTACTGGTCCT
GTTATGTGCACTTGCAGCTGCAGATGC* (SEQ ID NO: 29)

[0190] 3' PR8 terminal sequence:

*ACAGATTCTGGCGATCTACTCAACTGTGCGCCAGTTCACTGGTGCTTTTGGTCTCCC
TGGGGGCAATCAGTTTCTGGATGTGTTCTAATGGATCTTTCAGTGCAGAAATATGCATC
TGAGATTAGAATTTTCAGAGATATGAGGAAAAACACCCTTGTTTCT* (SEQ ID NO: 30)

[0191] The optimized P1 COBRA sequence was further modified by swapping 5' and 3' termini with the COBRA A1 sequence (see Table 12). More specifically, the 5' nucleotide sequence encoding the signal peptide of COBRA P1 was swapped with the corresponding COBRA A1 sequence, resulting in amino acid changes in the signal peptide. The 3' nucleotide sequence from COBRA P1 was also exchanged with the corresponding sequence from COBRA A1, including the sequence encoding the transmembrane region. However, this exchange did not introduce any amino acid changes in the 3' terminus. The 5' and 3' COBRA A1 terminal sequences that were exchanged correspond to SEQ ID NO: 31 and SEQ ID NO: 32, respectively. The coding sequences are italicized.

[0192] 5' COBRA A1 terminal sequence:

*ATGAAAGCAAACTACTAGTTCTGTTATGTGCATTTACAGCTACATATGCAGACACAATA
TGTATAGGCTACCATGCGAACAACCTCAACCGACACTGTTGACACAGTACTTGAAAAGAA
CGTGACAGTGACACACTCTGTCAACCTACTTGAGGACAGTCACAACGGAAACTA*
(SEQ ID NO: 31)

[0193] 3' COBRA A1 terminal sequence:

*GTGAAAAATGGAACCTTATGACTATCCAAAATATTCCGAGGAATCAAAGTTAAACAGGGA
AAAAATTGATGGAGTGAAATTGGAATCAATGGGAGTCTATCAGATTCTGGCGATCTACT
CAACTGTGCGCCAGTTCACTGGTGCTTTTGGTCTCCCTGGGGGCAATCAGCTTCTGGAT
GTGTTCTAATGGGTCTTTCAGTGTAGAAATATGCATCTGAGATTAGAATTTTCAGAGAT
ATGAGGAAAAACACCCTTGTTTCT* (SEQ ID NO: 32)

[0194] These additional optimized X6, X1, and P1 sequences are identified in Table 16 below as “codon bias + swap (termini or signal peptide).” All optimized nucleotide sequences were cloned by homologous recombination into a reverse genetics plasmid (“optimized HA plasmid”). The X6 codon bias sequence could not be cloned into the reverse genetics plasmid due to instability in *E. coli*. Viral rescue or recovery was tested in a reverse genetics system by co-transfecting into a mixed 293FT/MDCK cell culture the optimized HA plasmid with an NA plasmid (encoding various NA proteins as indicated in Table 16 below) and a PR8 backbone plasmid. Virus recovery was monitored for up to 10 days by measuring HA activity of the cell culture supernatant. HA titer was determined using turkey red blood cells and was calculated as the reciprocal of the highest viral suspension dilution with HA activity. Recovered virus was harvested from the cell culture and used to inoculate 10-day old hen embryonated eggs and viral growth was determined 72 hours post-inoculation.

[0195] All vaccine candidates were successfully recovered as viruses with at least one of the optimized nucleotide sequences generated with this new methodology, as summarized below in Table 16. In most cases, viruses recovered from cell culture were also able to grow in eggs at high titers ($>1 \times 10^6$ pfu/ml), thereby showing promise as seeds for vaccine manufacturing in eggs. The COBRA P1 codon bias sequence (without additional 5’ or 3’ termini swap) was able to support viral rescue in cell culture and eggs with some, but not all NAs tested. Thus, in certain instances, the codon bias sequence was sufficient to support viral rescue. Swapping the termini of the optimized P1 sequence resulted in viral rescue for all NAs tested. For the X1 and X6 optimized sequences, codon bias alone was not sufficient to support viral rescue. However, exchanging the 5’ and 3’ coding sequences (e.g., signal peptide, transmembrane and/or cytoplasmic domain) of the codon bias sequences, permitted viral recovery both in cell culture and in eggs.

[0196] Table 16

Hemagglutinin (HA) candidate	HA Nucleotide sequence generation	Neuraminidase	Virus recovery in 293FT/MDCK cells	Virus passage in hen embryonated eggs	
			HA titer	HA titer	Plaque assay
COBRA P1	Codon bias	N3SB-DB06	ND		
		N3TK-IT02	ND		
		N1_FortMontmouth47	ND		
		N1_Singapore86	16	512	23 x 10 ⁶ pfu/ml
		N1_NewCaledonia99	8	512	13 x 10 ⁶ pfu/ml
		N1_California09	ND		
		N3SB-DB06	16	256	66 x 10 ⁶ pfu/ml
		N3TK-IT02	16	256	520 x 10 ⁶ pfu/ml

	Codon bias + termini swap with COBRA A1 virus	N1_FortMontmouth47	32	512	35 x 10 ⁶ pfu/ml
		N1_Singapore86	32	256	2.7 x 10 ⁶ pfu/ml
		N1_NewCaledonia99	32	256	1.1 x 10 ⁶ pfu/ml
		N1_California09	32	1024	3.7 x 10 ⁶ pfu/ml
COBRA X6	Codon bias				
	Codon bias + signal peptide swap with COBRA X3 virus	N3TK-IT02	32	512	1.3 x 10 ⁷ pfu/ml
		N1_California09	2		
	Codon bias + signal peptide swap with wild-type virus	N3SB-DB06	32	512	1.13 x 10 ⁷ pfu/ml
		N3TK-IT02	32	512	4.95 x 10 ⁷ pfu/ml
		N1_Singapore86	16	1024	1.98 x 10 ⁷ pfu/ml
		N1_NewCaledonia99	16	512	5.0 x 10 ⁶ pfu/ml
		N1_California09	8	256	1.20 x 10 ⁷ pfu/ml
COBRA X1	Codon bias	N3SB-DB06	ND		
		N3TK-IT02	ND		
		N1_PuertoRico34	ND		
		N1_New Jersey76	ND		
		N1_Fort Monmouth47	ND		
		N1_Boston	ND		
		N1_Singapore86	ND		
		N1_NewCaledonia99	ND		
		N1_California09	ND		
	Codon bias + termini swap with COBRA A1 virus	N3TK-IT02	1	256	>1.0 x 10 ⁵ pfu/ml
		N1_California09	ND	ND	
		N1_Singapore86	ND	512	>1.0 x 10 ⁶ pfu/ml
	Codon bias + termini swap with PR8 virus	N3TK-IT02	8	512	1.25 x 10 ⁶ pfu/ml
		N1_California09	ND	512	3.5 x 10 ⁴ pfu/ml
		N1_Singapore86	2	128	1.5 x 10 ⁶ pfu/ml

[0197] Example 2 – Nucleotide Sequence Optimization of Influenza B SMART HAs

[0198] Methods of generating an optimized nucleotide sequence encoding an engineered influenza structural protein were implemented using the following influenza B SMART HA polypeptides: br08_CO1 (SEQ ID NO: 75), br08_DO2 (SEQ ID NO: 76), br08_DO3 (SEQ ID NO: 77), pan90_DO2 (SEQ ID NO: 78), and ma12_RA82 (SEQ ID NO: 79). For each SMART HA, two optimized nucleotide sequences were produced: one that was obtained following steps 1-3b in Figure 1 and one that was obtained following steps 1-5 in Figure 1.

[0199] More specifically, for each of the br08_CO1, br08_DO2, br08_DO3, pan90_DO2 and ma12_RA82 SMART HAs, an optimized nucleotide sequence was obtained by reverse translating the SMART HA amino acid sequence, comparing the reverse translated nucleotide sequence to a database of influenza sequences, and optimizing the reverse translated nucleotide sequence according to the rules set forth in Steps 3a and 3b of Figure 1. The optimized nucleotide sequences were also modified by adding the 5' and 3' non-coding regions from successfully rescued strain B/Memphis/12/1997.

[0200] In the case of B/Memphis/12/1997, the following 5'- and 3'-terminal nucleotide sequences were used:

[0201] 5' B/Memphis/12/1997 terminal sequence

[0202] AGCAGAAGCAGAGCATTTTCTAATATCCACAAAATG (SEQ ID NO: 103)

[0203] 3' B/Memphis/12/1997 terminal sequence

[0204] TAAGGAAAATTAAGCCCTGTATTTTCCTTTATTGTAGTGCTTGTTTGCTTGT
TATCATTACAAAGAAACGTTATTGAAAAATGCTCTTGTTACTACT (SEQ ID NO:
104)

[0205] The optimized br08_CO1 SMART HA sequence was further modified by swapping 5' and 3' termini with the 5' and 3' termini of B/Brisbane/60/2008. More specifically, the 5' nucleotide sequence encoding the signal peptide and into an initial part of the ectodomain were swapped with the corresponding B/Brisbane/60/2008 sequence. This exchange did not introduce changes in the signal peptide or the ectodomain region (i.e., only codon changes were made). The 3' terminal region, encoding a portion of the ectodomain, transmembrane domain and cytoplasmic tail, was also swapped with the corresponding sequence from B/Brisbane/60/2008 without introducing changes in the protein coding sequence. In the case of conflicts the original codon was used. The 5' and 3' B/Brisbane/60/2008 terminal sequences that were exchanged correspond to SEQ ID NO: 105 and SEQ ID NO: 106, respectively:

[0206] 5' B/Brisbane/60/2008 terminal sequence:

[0207] ATGAAGGCAATAATTGTACTACTCATGGTAGTAACATCCAATGCAGATCG
AATCTGCACTGGGATAACATCGTCA (SEQ ID NO: 105)

[0208] 3' B/Brisbane/60/2008 terminal sequence:

[0209] GCAGGAGAATTTTCTCTCCCCACCTTTGATTCACCTGAATATTACTGCTGCA
TCTTTAAATGACGATGGATTGGATAATCATACTATACTGCTTTACTACTCAACTGC
TGCTCCAGTTTGGCTGTAACACTGATGATAGCTATCTTTGTTGTTTATATGGTCT
CCAGAGACAATGTTTCTTGCTCCATCTGTCTATAA (SEQ ID NO: 106)

[0210] The optimized br08_D02 SMART HA sequence was further modified by swapping 5' and 3' termini with the 5' and 3' termini of B/Brisbane/60/2008. More specifically, the 5' nucleotide sequence encoding the signal peptide and into an initial part of the ectodomain were swapped with the corresponding B/Brisbane/60/2008 sequence. This exchange did not introduce changes in the signal peptide or the ectodomain region (i.e., only codon changes were

made). The 3' terminal region, encoding a portion of the ectodomain, transmembrane domain and cytoplasmic tail, was also swapped with the corresponding sequence from B/Brisbane/60/2008 without introducing changes in the protein coding sequence. In the case of conflicts the original codon was used. The 5' and 3' B/Brisbane/60/2008 terminal sequences that were exchanged correspond to SEQ ID NO: 105 and SEQ ID NO: 106, respectively.

[0211] The optimized br08_DO3 SMART HA sequence was further modified by swapping 5' and 3' termini with the 5' and 3' termini of B/Brisbane/60/2008. More specifically, the 5' nucleotide sequence encoding the signal peptide and into an initial part of the ectodomain were swapped with the corresponding B/Brisbane/60/2008 sequence. This exchange did not introduce changes in the signal peptide or the ectodomain region (i.e., only codon changes were made). The 3' terminal region, encoding a portion of the ectodomain, transmembrane domain and cytoplasmic tail, was also swapped with the corresponding sequence from B/Brisbane/60/2008 without introducing changes in the protein coding sequence. In the case of conflicts the original codon was used. The 5' and 3' B/Brisbane/60/2008 terminal sequences that were exchanged correspond to SEQ ID NO: 105 and SEQ ID NO: 106, respectively.

[0212] The optimized pan90_DO2 SMART HA sequence was further modified by swapping 5' and 3' termini with the 5' and 3' termini of B/Brisbane/60/2008. More specifically, the 5' nucleotide sequence encoding the signal peptide and into an initial part of the ectodomain were swapped with the corresponding B/Brisbane/60/2008 sequence. This exchange did not introduce changes in the signal peptide or the ectodomain region (i.e., only codon changes were made). The 3' terminal region, encoding a portion of the ectodomain, transmembrane domain and cytoplasmic tail, was also swapped with the corresponding sequence from B/Brisbane/60/2008 without introducing changes in the protein coding sequence. In the case of conflicts the original codon was used. The 5' and 3' B/Brisbane/60/2008 terminal sequences that were exchanged correspond to SEQ ID NO: 105 and SEQ ID NO: 106, respectively.

[0213] The optimized ma12_RA82 SMART HA sequence was further modified by swapping 5' and 3' termini with the 5' and 3' termini of B/Brisbane/60/2008. More specifically, the 5' nucleotide sequence encoding the signal peptide and into an initial part of the ectodomain were swapped with the corresponding B/Brisbane/60/2008 sequence. This exchange did not introduce changes in the signal peptide or the ectodomain region (i.e., only codon changes were made). The 3' terminal region, encoding a portion of the ectodomain, transmembrane domain and cytoplasmic tail, was also swapped with the corresponding sequence from B/Brisbane/60/2008 without introducing changes in the protein coding sequence. In the case

of conflicts the original codon was used. The 5' and 3' B/Brisbane/60/2008 terminal sequences that were exchanged correspond to SEQ ID NO: 105 and SEQ ID NO: 106, respectively.

[0214] Viral recovery experiments with the optimized nucleic acids derived from the influenza B SMART HAs have not yet been tested.

[0215] Example 3 – Nucleotide Sequence Optimization of H3 COBRAs

[0216] Methods of generating an optimized nucleotide sequence encoding an engineered influenza structural protein were implemented using 6 different H3 COBRAs. For each H3 COBRA HA polypeptide, an optimized nucleotide sequence was obtained by following steps 1-3b in Figure 1. The optional steps 4 and 5 were not carried out for these polypeptides.

[0217] More specifically, for each of the H3 COBRAs, an optimized nucleotide sequence was obtained by reverse translating the H3 COBRA amino acid sequence, comparing the reverse translated nucleotide sequence to a database of influenza sequences, and optimizing the reverse translated nucleotide sequence according to the rules set forth in Steps 3a and 3b of Figure 1.

[0218] The optimized H3 COBRA nucleotide sequences were cloned by homologous recombination into a reverse genetics plasmid ("optimized HA plasmid"). Viral rescue or recovery was tested in a reverse genetics system by co-transfecting into a mixed 293FT/MDCK cell culture the optimized HA plasmid with an NA plasmid and a PR8 backbone plasmid. Virus recovery was monitored for up to 10 days by measuring HA activity of the cell culture supernatant. HA titer was determined using turkey red blood cells and was calculated as the reciprocal of the highest viral suspension dilution with HA activity. Recovered virus was harvested from the cell culture and used to inoculate 10-day old hen embryonated eggs and viral growth was determined 72 hours post-inoculation.

[0219] All of the optimized nucleotide sequences derived from the H3 COBRA polypeptides were successfully recovered as viruses with at least one of the optimized nucleotide sequences generated with this new methodology.

EQUIVALENTS

[0220] Use of ordinal terms such as "first," "second," "third," etc., in the claims to modify a claim element does not by itself connote any priority, precedence, or order of one claim element over another or the temporal order in which acts of a method are performed, but are used merely

as labels to distinguish one claim element having a certain name from another element having a same name (but for use of the ordinal term) to distinguish the claim elements.

[0221] The articles “a” and “an” as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to include the plural referents. Claims or descriptions that include “or” between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention also includes embodiments in which more than one, or the entire group members are present in, employed in, or otherwise relevant to a given product or process. Furthermore, it is to be understood that the invention encompasses all variations, combinations, and permutations in which one or more limitations, elements, clauses, descriptive terms, etc., from one or more of the listed claims is introduced into another claim dependent on the same base claim (or, as relevant, any other claim) unless otherwise indicated or unless it would be evident to one of ordinary skill in the art that a contradiction or inconsistency would arise. Where elements are presented as lists, (e.g., in Markush group or similar format) it is to be understood that each subgroup of the elements is also disclosed, and any element(s) can be removed from the group. It should be understood that, in general, where the invention, or aspects of the invention, is/are referred to as comprising particular elements, features, etc., certain embodiments of the invention or aspects of the invention consist, or consist essentially of, such elements, features, etc. For purposes of simplicity those embodiments have not in every case been specifically set forth in so many words herein. It should also be understood that any embodiment or aspect of the invention can be explicitly excluded from the claims, regardless of whether the specific exclusion is recited in the specification. The publications, websites and other reference materials referenced herein to describe the background of the invention and to provide additional detail regarding its practice are hereby incorporated by reference.

We claim:

1. A method of generating an optimized nucleotide sequence encoding an engineered influenza structural protein, the method comprising:
 - a) providing an amino acid sequence of the engineered influenza structural protein;
 - b) reverse-translating the amino acid sequence to generate a first nucleotide sequence that is a non-optimized parental nucleotide sequence;
 - c) identifying a second nucleotide sequence that encodes an influenza structural protein that shares at least 90% sequence identity with the engineered influenza structural protein, which first and second nucleotide sequences comprise one or more positions where codons are different from one another;
 - d) at every position where the codons in the first and second nucleotide sequences code for the same amino acid, changing codons in the first nucleotide sequence to match codons from the second nucleotide sequence; and
 - e) at every position where the codons in the first and second nucleotide sequences code for a different amino acid, changing codons in the first nucleotide sequence to match codons that are based on structural protein-specific influenza codon usage preferences to generate the optimized nucleotide sequence, wherein translation of the optimized nucleotide sequence and expression of the encoded engineered influenza structural protein are improved for one or more expression systems relative to the non-optimized parental nucleotide sequence.
2. The method of claim 1, wherein the influenza structural protein that shares a high degree of sequence identity with the engineered influenza structural protein is a wild-type influenza structural protein.
3. The method of claim 1 or 2, further comprising adding the 5' and 3' non-coding sequences from a high titer rescued strain to the optimized nucleotide sequence.
4. The method of claim 3, wherein in the high titer rescued strain is A/PuertoRico/8/34 (PR8).

5. The method of anyone of claims 1-4, wherein the amino acid sequence of the engineered influenza structural protein encoded by the optimized nucleotide sequence is the same as the amino acid sequence encoded by the first nucleotide sequence.
6. The method of any one of claims 1-5, wherein the optimized nucleotide sequence further comprises a nucleotide sequence encoding a signal peptide, a nucleotide sequence coding for a transmembrane domain, and/or a nucleotide sequence coding for a cytoplasmic domain.
7. The method of claim 6, further comprising exchanging the nucleotide sequence encoding the signal peptide in the optimized nucleotide sequence with a nucleotide sequence encoding the signal peptide from a high titer rescued strain.
8. The method of claim 6 or 7, further comprising exchanging the nucleotide sequence encoding the transmembrane domain with a nucleotide sequence encoding the transmembrane domain from a high titer rescued strain.
9. The method of any one of claims 6-8, further comprising exchanging the nucleotide sequence encoding the cytoplasmic domain with a nucleotide sequence encoding the cytoplasmic domain from a high titer rescued strain.
10. The method of any one of claims 7-9, wherein the high titer rescued strain is A/PuertoRico/8/34 (PR8).
11. The method of any one of the preceding claims, where in the engineered influenza structural protein is an influenza type A hemagglutinin protein.
12. The method of claim 11, wherein the hemagglutinin protein is a subtype selected from the group consisting of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, and H17.

13. The method of any one of the preceding claims, wherein the structural protein-specific influenza codon usage preferences are set forth in Tables 1-10.

14. The method of any one of the preceding claims, wherein reverse translating the amino acid sequence to generate a first nucleotide sequence comprises use of codon usage preferences specific for influenza viruses.

15. The method of any one of the preceding claims, wherein the second nucleotide sequence encodes a wild type version of the influenza structural protein and is identified from a publicly available database comprising influenza nucleotide sequences.

16. The method of claim 3, wherein the 5' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 23 and/or the 3' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 24 or wherein the 5' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 103 and/or the 3' non-coding sequence comprises the nucleotide sequence of SEQ ID NO: 104.

17. The method of any one of the proceeding claims, wherein the engineered influenza structural protein comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, and SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 75, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 83, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, SEQ ID NO: 91, SEQ ID NO: 92, SEQ ID NO: 93, SEQ ID NO: 94, SEQ ID NO: 95, SEQ ID NO: 96, SEQ ID NO: 97, SEQ ID NO: 98, SEQ ID NO: 99, SEQ ID NO: 100, SEQ ID NO: 101, and SEQ ID NO: 102.

18. A method of expressing an optimized nucleotide sequence, the method comprising:
generating an optimized nucleotide sequence encoding an engineered influenza structural protein according to the method of any one of claims 1-17;

inserting the optimized nucleotide sequence into an expression plasmid; and
expressing the optimized nucleotide sequence to generate the engineered influenza structural protein.

19. A reverse genetics method for producing an infectious influenza virus, the method comprising:

(i) generating an optimized nucleotide sequence encoding an engineered influenza structural protein according to the method of any one of claims 1 to 17;

(ii) transfecting mammalian cells with one or more expression vectors, wherein the one or more expression vectors comprise: a) the optimized nucleotide sequence encoding the engineered influenza structural protein generated in step (i) and b) nucleotide sequences coding for influenza proteins from one or more donor viruses; and,

(iii) producing the infectious influenza virus.

20. The method of claim 19, wherein the one or more donor viruses are selected from the group consisting of A/Puerto Rico/8/34 (H1N1) (PR8), B/Lee/40, and B/Panama/45/90.

21. The method of claim 19 or 20, wherein the infectious influenza virus is an infectious reassortant influenza virus comprising the genetic material of one or more donor viruses.

22. The method of claim 21, wherein the infectious reassortant influenza virus is chimeric.

23. The method of any one of claims 19-22, further comprising:

harvesting the infectious influenza virus; and

infecting eggs or mammalian cells with the harvested influenza virus.

24. A method of preparing an influenza vaccine composition, the method comprising:

(i) generating an optimized nucleotide sequence encoding an engineered influenza structural protein according to the method of any one of claims 1 to 17;

(ii) generating a seed virus by transfecting mammalian cells with a set of expression vectors, one or more of which comprises the optimized nucleotide sequence encoding the engineered influenza structural protein generated in step (i);

(iii) harvesting the seed virus; and
producing infectious influenza virus by infecting eggs or mammalian cells with the seed virus;

(iv) harvesting the infectious influenza virus after multiplication in the eggs or mammalian cells;

(v) purifying the harvested infectious influenza virus;

(vi) optionally inactivating the purified virus; and

(vii) mixing the purified virus with a pharmaceutically acceptable carrier.

25. An influenza vaccine composition for use in inducing an immune response to one or more influenza polypeptides in a subject,

wherein the influenza vaccine composition comprises a purified infectious influenza virus that comprises an optimized nucleotide sequence encoding an engineered influenza structural protein that comprises at least one amino acid difference from an engineered influenza structural protein encoded by a non-optimized parental nucleotide sequence of the optimized nucleotide sequence;

wherein the engineered influenza structural protein encoded by the non-optimized parental nucleotide sequence shares at least 90% sequence identity with an influenza structural protein encoded by a second nucleotide sequence, which non-optimized parental nucleotide and second nucleotide sequences comprise one or more positions where codons are different from one another;

wherein the optimized nucleotide sequence comprises codons that match codons from the second nucleotide sequence at every position where the codons in the non-optimized parental nucleotide and second nucleotide sequences code for the same amino acid;

wherein the optimized nucleotide sequence comprises codons that match codons that are based on structural protein-specific influenza codon usage preferences at every position where the codons in the non-optimized parental nucleotide and second nucleotide sequences code for a different amino acid; and,

wherein translation of the optimized nucleotide sequence and expression of the encoded engineered influenza structural protein are improved for one or more expression systems relative to the non-optimized parental nucleotide sequence.