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(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA,

(54) **Title:** CHIMERIC INFLUENZA A VIRUS NEURAMINIDASE PROTEINS, VIRUS LIKE PARTICLES COMPRISING SAME, AND METHODS OF USE THEREOF

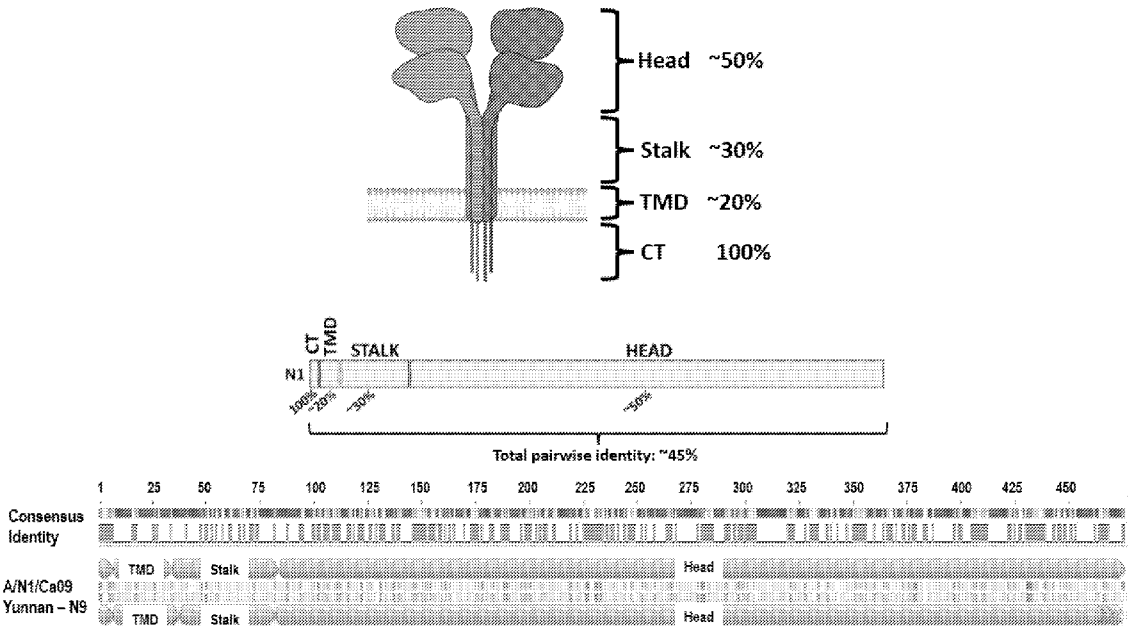


FIG. 1D

(57) **Abstract:** The present disclosure relates to a chimeric influenza A virus (IAV) neuraminidase (NA) protein comprising: a cytoplasmic tail; a transmembrane domain; a stalk domain; and a head domain, where the cytoplasmic tail, the stalk domain, and the head domain are from a first influenza A virus neuraminidase protein, where the transmembrane domain is from a second influenza A virus neuraminidase protein which is different from the first influenza A virus, said second influenza A virus neuraminidase protein having a budding phenotype which is greater than the budding phenotype of the first influenza A virus neuraminidase protein. Also disclosed are nucleic acid molecules encoding the chimeric IAV NA, vectors comprising said nucleic acid molecules, host cells comprising said vectors, virus-like particles (VLPs) comprising the chimeric IAV NA, methods of producing VLPs, and methods of eliciting an immune

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CHIMERIC INFLUENZA A VIRUS NEURAMINIDASE PROTEINS, VIRUS LIKE PARTICLES COMPRISING SAME, AND METHODS OF USE THEREOF

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[0001] This application claims the benefit of U.S. Provisional Patent Application Serial No. 63/433,654, filed December 19, 2022, which is hereby incorporated by reference in its entirety.

10 [0002] This invention was made with government support under R21AI156731 awarded by National Institutes of Health. The government has certain rights in the invention.

FIELD

[0003] The present disclosure relates to chimeric influenza A virus (IAV) neuraminidase (NA) proteins, as well as virus like particles comprising chimeric influenza A virus (IAV) neuraminidase (NA) proteins, and methods of use thereof.

BACKGROUND

[0004] Influenza A viruses (IAVs) are enveloped, single-stranded, negative sense RNA viruses with a segmented genome in the family *Orthomyxoviridae*. There are 8 segments which encode for at least 11 proteins (Krammer et al., “Influenza,” *Nat. Rev. Dis. Primers* 4(1):3 (2018)). Influenza viruses contain two major glycoproteins: hemagglutinin (HA) and neuraminidase (NA). IAVs are responsible for annual epidemics and occasional global pandemics. An estimated 3–5 million severe infections and 290,000–650,000 deaths occur every year due to seasonal influenza. In addition to these seasonal outbreaks, influenza pandemics pose a major threat to global health, which arise when novel proteins are introduced into naïve populations. These have historically been caused by IAVs from avian or swine species spilling over into humans. Current vaccines provide protection against seasonal strains but not for pandemic strains.

[0005] Most influenza vaccines currently administered are whole-inactivated virus (WIV). Candidate vaccine viruses (CVVs) are selected based on large-scale surveillance predictions of which strains are likely to be circulating in the next year. These selected strains are inoculated and grown in egg culture and require inactivation, most commonly with either formaldehyde or β -propiolactone. Egg-based WIV vaccines are on average only 40% effective (Zimmerman et al., “2014-2015 Influenza Vaccine Effectiveness in the United States by Vaccine

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Type,” *Clin. Infect. Dis.* 63(12):1564-1573 (2016)). This low efficacy stems from mismatches in the prediction of vaccine strains against circulating strains (Krammer and Palese, “Advances in the Development of Influenza Virus Vaccines,” *Nat. Rev. Drug Discov.* 14(3):167-182 (2015)). Although HA is the main immunodominant target in live-virus infections and in current WIV
5 vaccines, it undergoes constant antigenic drift resulting in immune responses which are significantly less effective to even closely related strains (Krammer and Palese, “Advances in the Development of Influenza Virus Vaccines,” *Nat. Rev. Drug Discov.* 14(3):167-182 (2015)). This results in seasonal vaccination being necessary to provide adequate protection for newly circulating strains.

10 **[0006]** Although HA and NA have roughly equivalent immunogenicity, HA is immunodominant to NA, likely due to variable ratios of HA to NA, ranging from 3:1 – 18:1 (Sultana et al., “Stability of Neuraminidase in Inactivated Influenza Vaccines,” *Vaccine* 32(19):2225-2230 (2014) and Getie-Kehtie et al., “Label-Free Mass Spectrometry-Based Quantification of Hemagglutinin and Neuraminidase in Influenza Virus Preparations and
15 Vaccines,” *Influenza Other Respir. Viruses* 7(4):521-530 (2013). Although immune responses to NA are sub-optimal, NA vaccination has demonstrated fully protective immune responses as well as protection against heterologous strains (Pliasis et al., “A Novel Neuraminidase Virus-Like Particle Vaccine Offers Protection Against Heterologous H3N2 Influenza Virus Infection in the Porcine Model,” *Front. Immunol.* 7:13:915364 (2022); Kim et al., “Universal Protection
20 against Influenza Viruses by Multi-Subtype Neuraminidase and M2 Ectodomain Virus-Like Particle,” *PLoS Pathog.* 18(8):e1010755 (2022); and Wohlbold et al., “Vaccination with Adjuvanted Recombinant Neuraminidase induces broad Heterologous, but not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice,” *mBio* 6(2):e02556 (2015)). This indicates that NA vaccination may be an attractive candidate for universal influenza
25 vaccinations.

[0007] NA is a tetrameric type II transmembrane protein with enzymatic activity which serves vital roles in infection to cleave sialic acid moieties at the cell surface and in mucus (Yang et al., “A Beneficiary Role for Neuraminidase in Influenza Virus Penetration through the Respiratory Mucus,” *PLoS One* 9(10):e10026 (2014); Cohen et al., “Influenza A Penetrates Host
30 Mucus by Cleaving Sialic Acids with Neuraminidase,” *Virol. J.* 22:10:321 (2013); and McAuley et al., “Influenza Virus Neuraminidase Structure and Functions,” *Front. Microbiol.* 29:10:39 (2019)). Of the 11 known serotypes of NA, few are of major concern. Currently, H1N1 and H3N2 are the only seasonally circulating serotypes in the human population. However, other serotypes have been known to be highly pathogenic in humans, most notably H5N1 and H7N9
35 highly pathogenic avian influenzas (HPAI). NA is composed of 4 major domains: the

cytoplasmic tail (CT), transmembrane domain (TMD), stalk, and head. The CT is short, only 6AA, but very highly conserved, being nearly identical in almost every strain regardless of serotype. Despite this, the CT play vital roles in assembly and viral morphology (Jin et al., “Influenza Virus Hemagglutinin and Neuraminidase Cytoplasmic Tails Control Particle Shape,” *EMBO J.* 16(6):1236-1247 (1997) and Zhang et al., “Influenza Virus Assembly and Lipid Raft Microdomains: A Role for the Cytoplasmic Tails of the Spike Glycoproteins,” *J. Virol.* 74(10): 4634-4344 (2000)). The TMD has multiple roles, notably involved in translocation signal, lipid rafts, and apical sorting (Barman and Nayak, “Analysis of the Transmembrane Domain of Influenza Virus Neuraminidase, A Type II Transmembrane Glycoprotein, for Apical Sorting and Raft Association,” *J. Virol.* 74(14):6538-6545 (2000)). The TMD and stalk regions of NA can vary considerably, even within serotypes. NA can form virus-like particles (VLPs) following transfection of plasmid DNA into cell culture, independent of other viral proteins (Yondola et al., “Budding Capability of the Influenza Virus Neuraminidase can be Modulated by Tetherin,” *J. Virol.* 85(6):2480-2491 (2011)). However, the major drivers of NA budding are not well understood. Recent studies have shown that animal models are afforded protection from both homologous and heterologous strains following NA vaccination (Pliasis et al., “A Novel Neuraminidase Virus-Like Particle Vaccine Offers Protection Against Heterologous H3N2 Influenza Virus Infection in the Porcine Model,” *Front. Immunol.* 7:13:915364 (2022); Kim et al., “Universal Protection against Influenza Viruses by Multi-Subtype Neuraminidase and M2 Ectodomain Virus-Like Particle,” *PLoS Pathog.* 18(8):e1010755 (2022); and Wohlbold et al., “Vaccination with Adjuvanted Recombinant Neuraminidase induces broad Heterologous, but not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice,” *mBio* 6(2):e02556 (2015)).

[0008] Major bottlenecks still need to be addressed to improve NA production. Most NA production methods rely on Sf9 cell culture, an insect cell line, which significantly increases production of NA VLPs (Thompson et al., “Critical Assessment of Influenza VLP Production in Sf9 and HEK293 Expression Systems,” *BMC Biotechnol.* 16:15:31 (2015)). However, proteins produced from insect cells have been demonstrated to lack similar post-transcriptional modifications (Liu et al., “Use of Baculovirus Expression System for Generation of Virus-Like Particles: Successes and Challenges,” *Protein Expr. Purif.* 90(2):104-116 (2013)). Proper glycosylation has been shown to be necessary for reliable and effective vaccinations, playing roles in localization, stability, folding, and immunogenicity (Liu et al., “Use of Baculovirus Expression System for Generation of Virus-Like Particles: Successes and Challenges,” *Protein Expr. Purif.* 90(2):104-116 (2013); Gavrilov et al., “Effects of Glycosylation on Antigenicity and Immunogenicity of Classical Swine Fever Virus Envelope Proteins,” *Virology* 420(2):135-145

(2011); and Dowling et al., "Influences of Glycosylation on Antigenicity, Immunogenicity, and Protective Efficacy of Ebola virus GP DNA Vaccines," *J. Virol.* 81(4):1821-1837 (2007)).

Indeed, it has been demonstrated that NAs with missing or improper glycosylation have significant functional changes (Li et al., "Glycosylation of Neuraminidase Determines the

Neurovirulence of Influenza A/WSN/33 Virus," *J. Virol.* 67(11):6667-6673 (1993) and Saito and Kawano, "Loss of Glycosylation at Asn144 Alters the Substrate Preference of the N8 Influenza A Virus Neuraminidase," *J. Vet. Med. Sci.* 59(10):923-926 (1997)). This represents a major flaw in the use and application of insect cell culture methods to produce mammalian vaccines.

[0009] The present disclosure is directed to overcoming these and other deficiencies in the art.

SUMMARY

[0010] A first aspect of the present disclosure relates to a chimeric influenza A virus (IAV) neuraminidase (NA) protein comprising: a cytoplasmic tail; a transmembrane domain; a stalk domain; and a head domain, where the cytoplasmic tail, the stalk domain, and the head domain are from a first influenza A virus neuraminidase protein, where the transmembrane domain is from a second influenza A virus neuraminidase protein which is different from the first influenza A virus, said second influenza A virus neuraminidase protein having a budding phenotype which is greater than the budding phenotype of the first influenza A virus neuraminidase protein.

[0011] Another aspect of the present disclosure relates to a nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein of the present disclosure.

[0012] Another aspect of the present disclosure relates to a vector encoding a chimeric influenza A virus neuraminidase protein, where the vector comprises the nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein according to the present disclosure.

[0013] Another aspect of the present disclosure relates to a host cell comprising a nucleic acid molecule according to the present disclosure. Also disclosed are virus-like particles produced by host cells according to the present disclosure.

[0014] Another aspect of the present disclosure relates to a virus-like particle comprising a chimeric influenza A virus neuraminidase protein according to the present disclosure.

[0015] Another aspect of the present disclosure relates to a method of producing a virus-like particle comprising a chimeric influenza A virus neuraminidase protein. This method involves providing the vector according to the present disclosure; transfecting a host cell with the vector; and culturing the host cell in a cell culture comprising a supernatant under conditions

effective to express the chimeric influenza A virus neuraminidase protein and allow budding of virus-like particles comprising tetramers of the chimeric influenza A virus neuraminidase proteins into the supernatant.

[0016] Another aspect of the disclosure relates to a method of producing a virus-like particle comprising a chimeric influenza A virus neuraminidase protein. This method involves providing the host cell according to the present disclosure; and culturing the host cell in a cell culture comprising a supernatant under conditions effective to express the chimeric influenza A virus neuraminidase protein and to allow budding of virus-like particles comprising tetramers of the chimeric influenza A virus neuraminidase proteins into the supernatant.

[0017] Another aspect of the present disclosure relates to a pharmaceutical composition comprising a plurality of virus-like particles according to the present disclosure and a pharmaceutically acceptable carrier.

[0018] Another aspect of the present disclosure relates to a method of eliciting an immune response in a subject. This method involves administering an effective amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure, where said administering is effective to elicit an immune response in the subject.

[0019] The Examples of the present disclosure investigate the expression, budding potential, strength of oligomerization, and trafficking of multiple serotypes of NA. A high-budding strain of NA from the N9 serotype is identified. Further, chimeric NA proteins are designed and it is observed that the transmembrane domain contributes to increasing the budding phenotype of a low-budding N1, which results in ~50-fold increased incorporation of NA into VLPs in mammalian cell culture, overcoming the bottlenecks of N1 VLP production.

BRIEF DESCRIPTION OF THE DRAWINGS

[0020] FIGS. 1A-1D demonstrate the budding properties of neuraminidase proteins. FIG. 1A is a schematic showing the domains of influenza neuraminidase (NA) protein. The FLAG-tag (DYKDDDDK (SEQ ID NO: 8)) was added at the C-terminus. FIG. 1B is a Western blot showing the purified VLP fraction and cell lysate (CL) of NAs expressed from transfected HEK293T cells. All samples were run through SDS-PAGE and Western blotted. Ca09 and PR8 are NAs derived from H1N1 viruses. X31, X47, and X79 are NAs derived from H3N2 viruses. N9 was derived from an H7N9 virus. Control used was an empty plasmid backbone (pCAGGS). FIG. 1C is a bar graph showing the quantification of all bands performed using densitometric analysis. Normalized band intensity of VLPs divided by the normalized band intensity of CL

was used to establish the budding index. Statistical analyses were performed with one-ANOVA and Dunnett's multiple comparisons test ($N = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $P < 0.0001$). FIG. 1D shows the % identities shared between the Ca09 N1 and A/Yunnan/0129/2017 (H7N9) N9 sequences.

5 **[0021]** FIGS. 2A-2D demonstrate that the high-budding phenotype of N9 can be conferred to N1 by swapping the TMD. FIG. 2A is a schematic showing the percent identities of domains between Ca09-N1 and N9. FIG. 2B is a schematic showing the wildtype (WT) and the design of chimeric NAs. FIG. 2C is a Western blot depicting N1 and N9 parents and chimeras. FIG. 2D is a bar graph showing the quantification of all bands performed using densitometric
10 analysis in which normalized band intensity of VLPs was divided by the normalized band intensity of CL to establish the budding index. Statistical analyses were performed with one-way ANOVA and Dunnett's multiple comparisons test ($N = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $P < 0.0001$).

[0022] FIGS. 3A-3B shows that cell-surface expression of N1 can be modulated by N9
15 domains in chimeras. FIG. 3A is a bar graphs showing relative CSE of NAs normalized to highest signal. FIG. 3B is a bar graph showing normalized band intensity of VLPs divided by the normalized CSE in FIG. 3A. Statistical analyses were performed with one-way ANOVA and Dunnett's multiple comparisons test ($N = 3$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $P < 0.0001$).

[0023] FIGS. 4A-4D demonstrate that stalk and TMD chimeras can change strength of
20 oligomerization. FIG. 4A is a Western blot of cell lysates of transfected cells collected 24 hours post transfection and analyzed via a non-denaturing Western blot. Strength of oligomers was determined by analyzing the amount of monomers (FIG. 4B), dimers (FIG. 4C), or tetramers (FIG. 4D) divided by the total amount of protein. Statistical analyses were performed with one-ANOVA and Dunnett's multiple comparisons test ($N = 5$; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$,
25 $P < 0.0001$).

DETAILED DESCRIPTION

[0024] Current influenza vaccines provide robust immune responses against
hemagglutinin but not against neuraminidase. The immunodominance of hemagglutinin (HA)
30 has several contributing factors, including higher amount of HA in viral particles, antigenic sin, and a lack of neuraminidase standardization in vaccines. Traditional influenza vaccines are whole inactivated viruses which are produced in eggs. HA is prone to antigenic drift, especially in the immunodominant head domain. This can contribute to mismatches between viral strains in vaccines and circulating viruses. Vaccines would benefit from increased immune responses

toward neuraminidase (NA), as NA is more highly conserved and less prone to antigenic drift, decreasing the impacts of vaccine mismatch.

[0025] Recent studies have shown that passive transfer of antibodies against NA alone can provide protection to both homologous and heterologous strains. However, protein production remains a major bottleneck for NA vaccines. Currently, insect cell culture has been used to overcome these production problems, however, insect cells deliver other drawbacks. There are major glycosylation differences between proteins made in mammalian cell culture and those made in insect cell culture, which can impact protein translocation, folding, antigenicity, and immunogenicity. Thus, methodology to increase production of NA in mammalian cell culture is highly desirable.

[0026] The Examples of the present disclosure identify a naturally high-budding strain of NA N9 and demonstrate that the transmembrane domain (TMD) of NA N9 can confer a high-budding phenotype to NA N1 without altering the strength of oligomers.

[0027] Accordingly, a first aspect of the present disclosure relates to a chimeric influenza A virus (IAV) neuraminidase (NA) protein comprising: a cytoplasmic tail; a transmembrane domain; a stalk domain; and a head domain, where the cytoplasmic tail, the stalk domain, and the head domain are from a first influenza A virus neuraminidase protein, where the transmembrane domain is from a second influenza A virus neuraminidase protein which is different from the first influenza A virus, said second influenza A virus neuraminidase protein having a budding phenotype which is greater than the budding phenotype of the first influenza A virus neuraminidase protein.

[0028] In some embodiments, the head domain is at the C-terminus of the neuraminidase protein, the head domain is connected at its amino end to the stalk domain of the neuraminidase protein, the stalk domain is connected at its amino end to the transmembrane domain, the transmembrane domain is connected at its amino end to the cytoplasmic domain, and/or the cytoplasmic domain is at the amino-terminus of the neuraminidase protein.

[0029] The term “subtype” refers to the different groupings of influenza A strains that can be divided and classified based on the HA and NA genes that are expressed in the virus strain. The influenza A subtype nomenclature is based on the HA subtype, *e.g.*, the subtype is any one of the 18 different HA genes known in the art (*i.e.*, H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, and H18), and the NA subtype, *e.g.*, any of the 11 different NA genes known in the art (*e.g.*, N1, N2, N3, N4, N5, N6, N7, N8, N9, N10, and N11). Exemplary influenza virus A subtypes include, but are not limited to, H1N1, H3N2, H7N9.

[0030] The term “strain” refers to the particular virus variant of a given species, *e.g.*, influenza A or B species, and subtype in an influenza A virus. For example, the virus A/California/7/2009 is an Influenza A virus, subtype H1N1, with the strain name A/California/7/2009.

5 **[0031]** Exemplary influenza A virus subtypes, strains, and neuraminidase proteins are shown in Table 1 below.

Table 1. Exemplary Neuraminidase Amino Acid Sequences

Description	Neuraminidase Amino Acid Sequence	SEQ ID NO:
A/Ohio/07/2009 (H1N1) N1 Neuraminidase GenBank Accession No.: ACP44181.1 “Ca09-N1”	MNPNQKIITI GSVCM TIGMA NLILQIGNII SIWISHSIQL GNQNQIETCN QSVITYENNT WVNQTYVNIS NTNFAAGQSV VSVKLAGNSS LCPVSGWAIY SKDNSIRIGS KGDV FVIREP FISCSPLECR TFFLTQ GALL NDKHSNGTIK DRSPYRTLMS CPIGEVPSPY NSRFESVAWS ASACHDGINW LTIGISGPDN GAVAVLKYNG IITDTIKSWR NNILRTQESE CACVNGSCFT VMTDGPSDGQ ASYKIFRIEK GKIVKSVEMN APNYHYEES CYPDSSEITC VCRDNWHGSN RPWVSFNQNL EYQIGYICSG IFGDNPRPND KTGSCGPVSS NGANGVKGFS FKYGNVWIG RTKSISSRNG FEMIWD PNGW TGTDN NFSIK QDIVGINEWS GYSGSFVQHP ELTG LDCIRP CFWVELIRGR PKENTIWTSG SSISFCGVNS DTVGWSWPDG AELPFTIDK	1
A/Puerto Rico/8/1934 (H1N1) N1 Neuraminidase GenBank Accession No.: ABD77678.1 “PR8-N1”	MNPNQKIITI GSICLVVGLI SLILQIGNII SIWISHSIQT GSQNHTGICN QNIITYKNST WVKDTTSVIL TGNSSLCPIR GWAIYSKDNS IRIGSKGDVF VIREPFISCS HLECR TFFLT QGALLNDKHS SGTVKDRSPY RALMSCPVGE APSPYNSRFE SVAWSASACH DGMGWLTIGI SGPDNGAVAV LKYNGIITET IKS WRKKILR TQESE CACVN GSCFTIMTDG PSDGLASYKI FKIEKGKVT K SIELNAPNSH YEECSCYPDT GKVMCVC RDN WHGSNRPWVS FDQNL DYQIG YICSGVFGDN PRPEDGTGSC GPVYVDGANG VKGFSYRYGN GVWIGRTKSH SSRHGFEMI W DPNGWTETDS KFSVRQDVVA MTDWSGYSGS FVQHPELTGL DCMRPCFWVE LIRGRPKEKT IWTSASSISF CGVNSDTVDW SWPDGAELPF SIDK	2
A/Hong Kong/1/1968 (H3N2) N2 Neuraminidase GenBank Accession No.: Q91MA2.1 “X31-N2”	MNPNQKIITI GSVSLTIATV CFLMQIAILV TTVTLHFKQY ECDSPASNQV MPCEPIIER NITEIVYLN T TIEKEICPK VVEYRNWSKP QCQITGFAPF SKDNSIRLSA GGDIWVTREP YVSCDHGKCY QFALGQG TTL DNKHSNDTIH DRIPHRTL LM NELGV PFHLG TRQVCIAWSS SSCHDGKAWL HVCITGDDKN ATASFIYDGR	3

	LVDSIGSWSQ NILRTQESEC VCINGTCTVV MTDGSASGRA DTRILFIEEG KIVHISPLSG SAQHVEECSC YPRYPGVRCI CRDNWKGSNR PVVDINMEDY SIDSSYVCSG LVGDTPRNDD RSSNSNCRNP NNERGNQGVK GWAFDNGDDV WMGRTISKDL RSGYETFKVI GGWSTPNSKS QINRQVIVDS DNRSGYSGIF SVEGKSCINR CFYVELIRGR KQETRVWWTS NSIVVFCGTS GTYGTGSWPD GANINFMPI	
A/X-47 (H3N2) N2 Neuraminidase GenBank Accession No.: AAB03361.1 “X47-N2”	MNPNQKIITI GSVSLTIATI CFLMQIAILV TTVTLHFQY ECDSPANNOV MPCEPIIER NITEIVYLTN TTIEKEICPK LVEYRNWSKP QCKITGFAPF SKDNSIRLSA GGDIWVTREP YVSCDPGKCY QFALGQGTTL ENKHSNDTIH DRTPHRTLLM NELGVPFHLG TRQVCIAWSS SSCHDGKAWL HVCVTGYDKN ATASFIYDGR LVDSIGSWSQ NILRTQESEC VCINGTCTVV MTDGSASGRA DTKILFIEEG KIVHISPLSG SAQHVEECSC YPRYPGVRCI CRDNWKGSNR PVVDINVKDY SIDSSYVCSG LVGDTPRKND RSSSSYCRNP NNEKGNHGVK GWAFDDGNDV WMGRTISEDS RSGYETFKVI GGWSTPNSKL QINRQVIVDS ANRSGYSGIF SVEGKSCINR CFYVELIRGR EQETRVWWTS NSIVVFCGTS GTYGTGSWPD GADINLMPI	4
A/Philippines/2/1982 (H3N2) N2 Neuraminidase GenBank Accession No.: ADJ41808.1 “X79-N2”	MNPNQKIITI GSVSLTIATI CFLMQIAILV TTVTLHFQY ECSSPPNNQV VPCEPIIER NITEIVYLTN TTIEKEICPK LVEYRNWSKP QCKITGFAPF SKDNSIRLSA GGDIWVTREP YVSCDPGKCY QFALGQGTTL DNKHSNDTIH DRTPYRTLLM NELGVPFHLG TRQVCIAWSS SSCHDGKAWL HVCITGYDKN ATASFIYDGR LVDSIGSWSK NILRTQESEC VCINGTCTVV MTDGSASERA DTKILFIEEG KIVHISPLSG SAQHVEECSC YPRYPGVRCV CRDNWKGSNR PVVDINVKDY SIVSSYVCSG LVGDTPRKND RSSSSYCRNP NNEKGNHGVK GWAFDDGNDV WMGRTISEES RSGYETFKVI GGWSTPNSKL QINRQVIVDS GNRSGYSGIF SVEGKSCINR CFYVELIRGR EQETRVWWTS NSIVVFCGTS GTYGTGSWPD GADINLMPI	5
A/Yunnan/0129/2017 (H7N9) N9 Neuraminidase GenBank Accession No.: ARG43209.1 “N9”	MNPNQKILCT SATAITIGAI AVLIGIANLG LNIGLHLKPG CNCSHSQPET TNSQTIINN YYNETNITNI QMEERTSRNF NNLTKGLCTI NSWHIYGKDN AVRIGESSDV LVTREPYVSC NPDECRFYAL SQGTTIRGKH SNGTIHDRSQ YRALISWPLS SPPTVYNSRV ECIGWSSTSC HDGKSRMSIC ISGPNNNASA VVWYNRRPVA EINTWARNIL RTQESECVCH NGICPVVFTD GPATGPADTR IYYFKEGKIL KWESLTGTAK HIEECSCYGE RTGITCTCKD NWQGSNRPVI QIDPVAMTHT SQYICSPVLT DSPRPNDPNM	6

	GKCNDPYPGN NNNGVKGFSY LDGANTWLGR TISTTSRSGY EMLKVPNALT DDRSKPIQGQ TIVSNADWSG YSGSFMDYWA EGDCYRACFY VELIRGRPKE DKVWWTNSI VSMCSSTEFL GQWNWPDGAK IEYFL	
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[0032] As used herein, the term “chimeric” refers to a molecule (e.g., a protein) that contains portions thereof, from at least two different proteins. For example, a chimeric influenza NA protein refers to a protein comprising portions of a NA protein (e.g., the cytoplasmic tail, stalk domain, and/or head domain of a different influenza protein) and at least a portion of a heterologous influenza NA protein (for example a portion comprising a transmembrane domain). Chimeric proteins as described herein may include wild-type sequences or mutant sequences in any one, some, or all portions of the protein.

[0033] The terms “chimeric neuraminidase” or “chimeric influenza A virus neuraminidase” or “chimeric IAV neuraminidase” are used interchangeably and refer to a chimeric neuraminidase protein comprising, e.g., a cytoplasmic tail, stalk domain, and head domain from a first influenza A virus neuraminidase protein and transmembrane domain from a second influenza A virus neuraminidase protein. In some embodiments, the second influenza virus neuraminidase protein may be from an IAV of a different subtype than the first influenza virus neuraminidase. For example, the chimeric neuraminidase may comprise a cytoplasmic tail, stalk domain, and head domain from a N1 neuraminidase protein and a transmembrane domain from a N9 neuraminidase protein.

[0034] FIGS. 1A-1C demonstrate the budding properties exemplary of N1, N2, and N9 neuraminidase proteins. As described *infra*, the budding index of a neuraminidase protein (e.g., a wildtype or chimeric influenza A virus neuraminidase protein) may be determined by assessing whole-cell expression of a neuraminidase protein from an expression construct in a suitable cell line (e.g., HEK293T cells). Whole-cell expression may be assessed by quantifying the amount of neuraminidase protein present in the cell lysate and virus like particle fraction of a cell culture, e.g., by densitometric analysis. A budding index may be calculated by, e.g., dividing the normalized band intensity of the VLPs fraction by the normalized band intensity of the CL fraction. FIG. 1C demonstrates that the budding index of an N9 neuraminidase from an H7N9 virus is more than 30-fold greater than the budding index of N1 neuraminidase proteins from Ca09 H1N1, PR8 H1N1, X31 H3N2, X47 H3N2, and X79 H3N2.

[0035] The term “budding phenotype” refers to the budding profile as determined by the amount of NA incorporated into virus-like particles relative to the expression in or on the cell. Methods of determining the budding phenotype of an influenza A virus neuraminidase protein are well known in the art and include, e.g., measurement of NA in cell culture media, in the cell,

or on the cell surface by methods such as Western blots and Coomassie blots (*see, e.g., Yondola et al., "Budding Capability of the Influenza Virus Neuraminidase Can Be Modulated by Tetherin," J. Virol.* 85(6):2480-2491 (2011), which is hereby incorporated by reference in its entirety).

- 5 **[0036]** The terms "low-budding neuraminidase protein" or "low-budding influenza virus A neuraminidase protein" are used interchangeably and refer to, *e.g.*, neuraminidase proteins having a low budding index such as a budding index of less than 5, less than 4, less than 3, less than 2, or less than 1. In some embodiments, the first influenza A virus neuraminidase protein is a low-budding neuraminidase protein. In accordance with such embodiments, the low-budding
- 10 neuraminidase protein may be a N1 neuraminidase protein. For example, the low-budding neuraminidase protein may be an N1 neuraminidase having the sequence of SEQ ID NO:1 or SEQ ID NO:7 (Table 2). In some embodiments, the low-budding neuraminidase protein is Ca09-N1.

Table 2. Exemplary Low-Budding N1 Neuraminidase Proteins

Description	Neuraminidase Amino Acid Sequence	SEQ ID NO:
A/Ohio/07/2009 (H1N1) N1 Neuraminidase GenBank Accession No.: ACP44181.1	MNPNQKIITI GSVCM TIGMA NLILQIGNII SIWISHSIQL GNQNQIETCN QSVITYENNT WVNQTYVNIS NTNFAAGQSV VSVKLAGNSS LCPVSGWAIY SKDNSIRIGS KGDV FVIREP FISCSPLECR TFFLTQGALL NDKHSNGTIK DRSPYRTLMS CPIGEVPSPY NSRFESVAWS ASACHDGINW LTIGISGPDN GAVAVLKYNG IITDTIKSWR NNILRTQESE CACVNGSCFT VMTDGPSDGQ ASYKIFRIEK GKIVKSVEMN APNYHYEES CYPDSSEITC VCRDNWHGNS RPWVSFNQNL EYQIGYICSG IFGDNPRPND KTGSCGPVSS NGANGVKGFS FKYGNVWIG RTKSISRNG FEMIWDPNGW TGTDNNFSIK QDIVGINEWS GYSGSFVQHP ELTGLDCIRP CFWVELIRGR PKENTIWTSG SSISFCGVNS DTVGWSWPDG AELPFTIDK	1
A/California/14/2009 (H1N1) N1 Neuraminidase GenBank Accession No.: ACQ76308.1	MNPNQKIITI GSVCM TIGMA NLILQIGNII SIWISHSIQL GNQNQIETCN QSVITYENNT WVNQTYVNIS NTNFAAGQSV VSVKLAGNSS LCPVSGWAIY SKDNSIRIGS KGDV FVIREP FISCSPLECR TFFLTQGALL NDKHSNGTIK DRSPYRTLMS CPIGEVPSPY NSRFESVAWS ASACHDGINW LTIGISGPDN GAVAVLKYNG IITDTIKSWR NNILRTQESE CACVNGSCFT VMTDGPSDGQ ASYKIFRIEK GKIVKSVEMN APNYHYEES CYPDSSEITC VCRDNWHGNS RPWVSFNQNL EYQIGYICSG IFGDNPRPND KTGSCGPVSS NGANGVKGFS FKYGNVWIG	1

	RTKSISSRNG FEMIWDPNGW TGTDDNNFSIK QDIVGINEWS GYSGSFVQHP ELTGLDCIRP CFWVELIRGR PKENTIWTSG SSISFCGVNS DTVGWSWPDG AELPFTIDK	
A/California/07/2009 (H1N1) N1 Neuraminidase GenBank Accession No.: YP_009118627.1	MNPNQKIITI GSVCM TIGMA NLILQIGNII SIWISHSIQL GNQNQIETCN QSVITYENNT WVNQTYVNIS NTNFAAGQSV VSVKLAGNSS LCPVSGWAIY SKDNSVRIGS KGDV FVIREP FISCSPLECR TFFLTQGALL NDKHSNGTIK DRSPYRTLMS CPIGEVPSPY NSRFESVAWS ASACHDGINW LTIGISGPDN GAVAVLKYN IITDTIKSWR NNILRTQESE CACVNGSCFT VMTDGPSNGQ ASYKIFRIEK GKIVKSVEMN APNYHYECS CYPDSSEITC VCRDNWHGNS RPWVSFNQNL EYQIGYICSG IFGDNPRPND KTGSCGPVSS NGANGVKGFS FKYGNGVWIG RTKSISSRNG FEMIWDPNGW TGTDDNNFSIK QDIVGINEWS GYSGSFVQHP ELTGLDCIRP CFWVELIRGR PKENTIWTSG SSISFCGVNS DTVGWSWPDG AELPFTIDK	7

[0037] The terms “medium-budding neuraminidase protein” or “medium-budding influenza virus A neuraminidase protein” are used interchangeably and refer to, *e.g.*, neuraminidase proteins having an intermediate budding index such as a budding index between 5 to 50, between 10 to 50, between 20 to 50, between 30 to 50, or between 40 to 50.

- 5 **[0038]** The terms “high-budding neuraminidase protein” or “high-budding influenza virus A neuraminidase protein” are used interchangeably and refer to, *e.g.*, neuraminidase proteins having a high budding index such as a budding index of at least 50, at least 60, at least 70, at least 80, at least 90, or at about 100. In some embodiments, the second influenza A virus neuraminidase protein is a medium-budding or high-budding neuraminidase protein. In
10 accordance with such embodiments, the medium-budding or high-budding neuraminidase protein may be a N9 neuraminidase protein. For example, the medium-budding or high-budding neuraminidase protein may be a N9 neuraminidase having the sequence of SEQ ID NO: 6.

- [0039]** In some embodiments, the chimeric influenza A virus neuraminidase protein comprises: a cytoplasmic tail having the amino acid sequence of SEQ ID NO: 25; a stalk domain
15 having the amino acid sequence of SEQ ID NO: 26; and a head domain having the amino acid sequence of SEQ ID NO: 27, as set forth in Table 3 below.

Table 3. Exemplary Chimeric N1 Neuraminidase Domain Sequences

Description	Neuraminidase Amino Acid Sequence	SEQ ID NO:
Cytoplasmic tail domain	MNPNQKI	25

Stalk domain	SHSIQLGNQNQ IETCNQSVIT YENNTWVNQT YVNISNTNFA AGQSVV	26
Head domain	SVKLAGNSSL CPVSGWAIYS KDNSVRIGSK GDV FVIREPF ISCSPLECRT FFLTQGALLN DKHSNGTIKD RSPYRTL MSC PIGEVPSPYN SRFESVAWSA SACHDGINWL TIGISGPDNG AVAVLKYNGI ITDTIKSWRN NILRTQESEC ACVNGSCFTV MTDGPSNGQA SYKIFRIEKG KIVKSVEMNA PNYHYEECSC YPDSSEITCV CRDNWHGSNR PWVSFNQNL EYQIGYICSGI FGDNPRPNDK TGSCGPVSSN GANGVKGFSE KYGNGVWIGR TKSISSRNGF EMIWDPNGWT GTDNNFSIKQ DIVGINEWSG YSGSFVQHPE LTGLDCIRPC FWVELIRGRP KENTIWTSGS SISFCGVNSD TVGWSWPDGA ELPFTIDK	27

[0040] In some embodiments, the second influenza A virus neuraminidase protein is an N9 neuraminidase protein. For example, the second neuraminidase protein may be from a N9 neuraminidase from A/Yunnan/0129/2017. In some embodiments, the transmembrane domain has an amino acid sequence of SEQ ID NO: 6, see Table 1. Thus, in some embodiments, the chimeric influenza A virus neuraminidase protein comprises a transmembrane domain having the amino acid sequence of SEQ ID NO: 28 (ILCTSATAITIGAI~~AVLIGIANLGLNIGLHL~~).

[0041] In some embodiments, the cytoplasmic tail, stalk domain, and head domain are from an influenza A virus N1 neuraminidase protein and the transmembrane domain is from a influenza A virus N9 neuraminidase protein. For example, the chimeric neuraminidase protein may have an amino acid sequence of SEQ ID NO: 29, as follows.

MNPNQK**ILCTSATAITIGAI~~AVLIGIANLGLNIGLHL~~**SHSIQLGNQNQIETCNQSVITYE
NNTWVNQTYVNISNTNFAAGQSVVSVKLAGNSSLCPVSGWAIYSKDNSIRIGSKGDV FVI
REPFISCSPLECRTFFLTQGALLNDKHSNGTIKDRSPYRTL MSCPIGEVPSPYNSRFESV
AWSASACHDGINWL TIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECACVNGS
CFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNYHYEECSCYPDSSEITCVCRDNWH
GSRNPWVSFNQNL EYQIGYICSGIFGDNPRPNDKTGSCGPVSSN GANGVKGFSE KYGNGVWIGR
WIGRTKSISSRNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHPELTGLDC
IRPCFWVELIRGRP KENTIWTSGSSISFCGVNSD TVGWSWPDGAELPFTIDK (SEQ ID
NO: 29; N9 TMD shown in bold italic)

[0042] In some embodiments, the chimeric influenza A virus neuraminidase protein has a budding phenotype which is greater than the budding phenotype of the first influenza A virus neuraminidase protein.

[0043] In some embodiments, the chimeric influenza A virus neuraminidase protein has a budding phenotype which is at least about 10-fold greater, at least about 20-fold greater, at least about 30-fold greater, at least about 40-fold greater, or at least about 50-fold greater than the budding phenotype of the first influenza A virus neuraminidase protein. In some embodiments,

the budding phenotype of a neuraminidase protein can be determined by calculating its budding index.

[0044] In some embodiments, the chimeric influenza A virus neuraminidase protein has a budding index which is greater than the budding index of the first influenza A virus

neuraminidase protein. For example, the budding index of the chimeric influenza A virus neuraminidase protein may be at least about 10-fold greater, at least about 20-fold greater, at least about 30-fold greater, at least about 40-fold greater, at least about 50-fold greater, at least about 60-fold greater, at least about 70-fold greater, at least about 80-fold greater, at least about 90-fold greater, or at least about 100-fold greater than the budding index of the first influenza A virus neuraminidase protein. In some embodiments, the chimeric influenza A virus neuraminidase protein has a budding index which is about the same as the budding index of the second influenza A virus neuraminidase protein. In some embodiments, the chimeric influenza A virus neuraminidase protein has a budding index which is greater than the budding index of the second influenza A virus neuraminidase protein.

[0045] The length of the NA stalk domain is known to have impacts on pathogenicity and immunogenicity (Blumenkrantz et al., “The Short Stalk Length of Highly Pathogenic Avian Influenza H5N1 Virus Neuraminidase Limits Transmission of Pandemic H1N1 Virus in Ferrets,” *J. Virol.* 87(19): 10539-10551 (2013) and Broecker et al., “Extending the Stalk Enhances Immunogenicity of the Influenza Virus Neuraminidase,” *J. Virol.* 93(18):e00840-19 (2019), which are hereby incorporated by reference in their entirety). For this reason, multiple lengths of the stalk domain in NA chimera models were investigated in the Examples described herewith.

[0046] In some embodiments, the stalk domain of the chimeric influenza A virus neuraminidase is a short stalk (SS) domain. For example, the chimeric neuraminidase short stalk domain may have an amino acid sequence of SEQ ID NO: 30

(KPGCNC SHSQPETTNTSQTII NNYYNETNITNIQMEERTS).

[0047] In some embodiments, the stalk domain of the chimeric influenza A virus neuraminidase comprises a long stalk (LS) domain. For example, the chimeric neuraminidase long stalk domain may have an amino acid sequence of SEQ ID NO: 31

(KPGCNC SHSQPETTNTSQTII NNYYNETNITNIQMEERTSRNFNNLTKGLCT).

Nucleic Acid Molecules

[0048] Provided are nucleic acid molecules encoding a chimeric influenza A virus neuraminidase protein of the present disclosure.

[0049] The terms “nucleic acid molecules” and “nucleotide sequences” include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), combinations of DNA

and RNA molecules or hybrid DNA/RNA molecules, and analogs of DNA or RNA molecules. Such analogs can be generated using, for example, nucleotide analogs, which include, but are not limited to, inosine or tritylated bases. Such analogs can also comprise DNA or RNA molecules comprising modified backbones that lend beneficial attributes to the molecules such as, for example, nuclease resistance or an increased ability to cross cellular membranes. The nucleic acids or nucleotide sequences can be single-stranded, double-stranded, may contain both single-stranded and double-stranded portions, and may contain triple-stranded portions, but preferably is double-stranded DNA.

[0050] In some embodiments, the nucleic acid molecule is a DNA molecule.

10 [0051] Suitable chimeric influenza A virus neuraminidase proteins of the present disclosure are described in more detail *supra*. Table 4 provides nucleic acid and amino acid sequences for various chimeric influenza A virus neuraminidase protein and influenza virus neuraminidase proteins described herein.

Table 4.

	Sequence	SEQ ID NO:
N1 A/California/07/2009(H1N1)		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGATCATCACGATTGGATCAGTTTGCATG ACAATCGGGATGGCAAATCTGATACTCCAAATTGGTAATATTATT AGTATTTGGATCTCCACAGCATCCAACTCGGCAACCAGAACCAA ATCGAAACGTGTAATCAATCTGTAATCACTTATGAAAATAACACG TGGGTGAATCAAACGTATGTGAATATCTCCAATACCAATTTTCGCT GCTGGGCAAAGTGTGGTAAGCGTCAAACTCGCTGGCAACTCAAGC CTCTGTCCAGTGAGCGGATGGGCCATCTATAGCAAAGATAATAGC ATCCGAATCGGCTCAAAGGGGACGTATTTGTCATCCGCGAACCC TTTATATCATGCTCACCACCTTGAGTGTCTGATACATTCTTCCTCACC CAGGGTGCCTTCTGAATGACAAACACTCCAATGGCACTATAAAA GACCGATCCCCCTATCGGACTCTGATGTCATGTCCAATTGGCGAA GTGCCTAGCCCGTATAATTACGATTTGAATCAGTAGCTTGGAGC GCTTCTGCTTGCCATGATGGTATAAATTGGCTTACAATCGGAATT TCCGGCCCCGATAATGGCGCTGTGGCGGTGCTCAAATACAATGGT ATCATCACCGATAACAATCAAGTCTTGGCGCAACAACATTCTCCGA ACCCAGGAAAGTGAGTGTGCATGCGTAAATGGTAGCTGTTTCACC GTCATGACGGACGGGCCTTCCGACGGCCAGGCATCATACAAAATC TTCCGCATCGAGAAAGGAAAGATCGTCAAAGTGTTGAAATGAAT GCTCCCAATTACCATTATGAAGAATGCAGCTGTTATCCCGATTCC TCTGAGATCACCTGCGTATGTAGGGATAATTGGCACGGGTCCAAT CGGCCGTGGGTTTCATTTAATCAGAACCTTGAGTATCAGATTGGT TACATCTGCTCTGGGATATTTGGTGATAATCCGCGACCAAATGAC AAAACAGGGTCTTGCGGCCAGTGAGTTCAAATGGCGCCAATGGT GTAAAAGGTTTCTCTTTCAAATACGGCAATGGCGTTTGGATTGGC AGGACCAAAGTATCAGCAGTAGGAATGGATTGAGATGATTTGG GATCCCAATGGTTGGACGGGGACTGATAATAATTTCTCCATTAAG CAAGATATCGTCGGCATTAACGAATGGAGCGGTTACTCCGGCTCC	33

	TTTGTCCAACATCCTGAACTGACCGGCCTCGATTGTATCCGTCCA TGTTCCTGGGTAGAACTCATCAGGGGTAGACCTAAAGAGAATACC ATTTGGACATCTGGTTCTAGCATCAGCTTTTGCGGTGTCAACTCA GATACAGTTGGATGGTCTTGGCCTGACGGGGCAGAATTGCCTTTT ACCATAGATAAG gattacaaggatgacgacgataag TAATAA	
Amio Acid Sequence	MNPNQKIITIGSVCMITIGMANLILQIGNIISIWISHSIQLGNQNO IETCNQSVITYENNTWVNQTYVNIISNTNFAAGQSVSVKLAGNSS LCPVSGWAIYSKDNSIRIGSKGDVVFVIREPFFISCSPLECRTFFLT QGALLNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWS ASACHDGINWLTIGISGPDNGAVAVLKYNIGIITDTIKSWRNNILR TQESEACVNGSCFTVMTDGPDSGQASYKIFRIEKGKIVKSVEMN APNYHYEEESCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIG YICSGIFGDNPRPNDKTGSCGPVSSNGANGVKGFSFKYGNVWIG RTKSISSRNGFEMIWDPNGTGTDDNNFSIKQDIVGINEWSGYSGS FVQHPFLTGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNS DTVGWSWPDGAELPFTIDK dykdddk **	34
N9 A/Yunnan/0129/2017 (H7N9)		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGAT CTCTGTGTACCTCCGCGACCGCTATC ACAATTGGGGCCATCGCCGTTCTGATCGGAATAGCAAACCTCGGA CTGAACATCGGACTTCATTTG <i>aagccaggatgtaattgcagtcac</i> <i>tctcagccccgagacaacaaacacgtcccaaaccattattaacaac</i> <i>tactataacgaaacaaatatcactaacattcagatggaggagaga</i> <i>acatctcgtaatctcaacaacctcacaaaaggactctgcaca</i> ATA AATTCCTGGCATATCTACGGCAAAGACAATGCCGTACGTATTGGC GAGAGCTCAGACGTTCTCGTAACGAGGGAACCTTATGTGAGTTGT AACCCTGACGAGTGTCGCTTCTACGCCCTCTCTCAAGGTACTACT ATCAGAGGTAAGCATTCTAATGGGACTATTTCATGATAGAAGCCAG TACCGGGCGTTGATTTCTGGCCGCTTAGCTCACCTCCGACCGTC TATAACTCTCGCGTGGAATGTATTGGCTGGTCCTCAACCAGCTGT CATGACGGGAAAAGTCGCATGTCTATTTGTATAAGTGGCCCGAAT AACAATGCTAGTGCCGTGGTTTGGTATAATCGTCGACCAGTGGCT GAAATAAACACTTGGGCCCCGAAATATCCTCCGCACCCAAGAGTCC GAATGTGTCTGTACAAACGGCATATGTCCTGTTGTCTTCACAGAC GGGCCGGCAACCGGTCTGCGGATACTCGAATCTATTACTTCAAG GAAGGTAAGATCCTCAAGTGGGAGAGTCTCACGGGGACAGCCAAG CACATTGAGGAGTGCAGCTGCTACGGTGAACGTACAGGCATAACA TGTACGTGCAAAGACAATTGGCAGGGGAGTAATAGGCCCGTTATC CAAATCGACCCGGTAGCCATGACTCATAACATCCAGTACATCTGT TCACCGGTTCTTACTGACAGTCCTCGACCCAACGACCCCAATATG GGGAAGTGTAACGACCCGTATCCCGGTAACAATAAACGGGGTT AAAGGCTTTTCTTACTTGGACGGCGCCAATACGTGGCTGGGCCGG ACCATCTCCACAACCTAGCAGATCCGGCTACGAGATGCTTAAGGTC CCCAACGCACTCACTGATGACCGTAGCAAGCCCATCCAAGGTCAA ACCATCGTGAGCAATGCAGACTGGTCTGGGTATAGCGGCTCCTTC ATGGACTACTGGGCTGAGGGAGACTGCTACAGAGCTTGTTTCTAT GTAGAAGTGAATTCGCGGCAGGCCAAAGGAGGACAAGGTGTGGTGG ACATCAAACCTCAATCGTGTCCATGTGCTCCTCTACCGAGTTTTTA GGGCAGTGGAAGTGGCCCGATGGGGCTAAGATAGAGTATTTCTTG gattacaaagacgatgatgacaag TAGTGA	35
Amino Acid Sequence	MNPNQK ILCTSATAITIGAIAVLIGIANLGLNIGLHL <i>kpgcncsh</i> <i>sqpettnsqtiinnyynetnitniqmeertsrnfnnltkglctI</i> NSWHIYGKDNAVRIGESSDVLVTREPYVSCNPDECRFYALSQGT	36

	IRGKHSNGTIHDRSQYRALISWPLSSPPTVYNSRVECIGWSSTSC HDGKSRMSICISGPNNNASAVVWYNRRPVAEINTWARNILRTQES ECVCHNGICPVVFTDGPATGPADTRIYYFKEGKILKWESLTGTAK HIEECSCYGERTGITCTCKDNWQGSNRPVIQIDPVAMTHTSQYIC SPVLTDSPRPNDPNMGKCNDPYPGNNNNGVKGFSYLDGANTWLGR TISTTSRSGYEMLKVPNALTDDRSPKPIQGQTIVSNADWSGYSGSF MDYWAEGDCYRACFYVELIRGRPKEDKVWWTSNSIVSMCSSTEFL GQWNWPDGAKIEYFLdykdddk**	
N9/N1 Chimera Long Stalk		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGATCATCACGATTGGATCAGTTTGCATG ACAATCGGGATGGCAAATCTGATACTCCAAATTGGTAATATTATT AGTATTTGGATCaagccaggatgtaattgcagtcattctcagccc gagacaacaaacacgtcccaaaccattattaacaactactataac gaaacaaatatcactaacattcagatggaggagagaacatctcgt aatctcaacaacctcacaaaaggactctgcacaGTGAGCGGATGG GCCATCTATAGCAAAGATAATAGCATCCGAATCGGCTCAAAGGG GACGTATTTGTCATCCGCGAACCCTTTATATCATGCTCACCATT GAGTGTCTGATACATTCTTCCTCACCCAGGGTGCGCTTCTGAATGAC AAACACTCCAATGGCACTATAAAAGACCGATCCCCCTATCGGACT CTGATGTCATGTCCAATTGGCGAAGTGCCTAGCCCGTATAATTCA CGATTTGAATCAGTAGCTTGGAGCGCTTCTGCTTGCCATGATGGT ATAAATTGGCTTACAATCGGAATTTCCGGCCCCGATAATGGCGCT GTGGCGGTGCTCAAATACAATGGTATCATCACCGATAACAATCAAG TCTTGGCGCAACAACATTCTCCGAACCCAGGAAAGTGAGTGTGCA TGCGTAAATGGTAGCTGTTTCACCGTCATGACGGACGGGCCTTCC GACGGCCAGGCATCATACAAAATCTTCCGCATCGAGAAAGGAAAG ATCGTCAAAGTGTTGAAATGAATGCTCCCAATTACCATTATGAA GAATGCAGCTGTTATCCCGATTCTCTGAGATCACCTGCGTATGT AGGGATAATTGGCACGGGTCCAATCGGCCGTGGGTTTCATTTAAT CAGAACCCTTGAGTATCAGATTGGTTACATCTGCTCTGGGATATTT GGTGATAATCCGCGACCAAATGACAAAACAGGGTCTTGCGGCCCA GTGAGTTCAAATGGCGCCAATGGTGTAAAAGGTTTCTCTTTCAA TACGGCAATGGCGTTTGGATTGGCAGGACCAAAAGTATCAGCAGT AGGAATGGATTTCGAGATGATTTGGGATCCCAATGGTTGGACGGGG ACTGATAATAATTTCTCCATTAAGCAAGATATCGTCGGCATTAAAC GAATGGAGCGGTTACTCCGGCTCCTTTGTCCAACATCCTGAACCTG ACCGGCCTCGATTGTATCCGTCCATGTTTCTGGGTAGAACTCATC AGGGGTAGACCTAAAGAGAATACCATTGGACATCTGGTTCTAGC ATCAGCTTTTGCGGTGTCAACTCAGATACAGTTGGATGGTCTTGG CCTGACGGGGCAGAATTGCCTTTTACCATAGATAAGgattacaag gatgacgacgataagTAATAA	37
	MNPNQKIITIGSVCMTIGMANLILQIGNIISIWIkpgcncshsqp ettntsqtiiannyynetnitiqmeertsrnfnnltkglctVSGW AIYSKDNSIRIGSKGDVVFVIREPFISCSPLECRTFFLTQGALLND KHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWSASACHDG INWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECA CVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVEMNAPNYHYE ECSCYPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICSGIF GDNPRPNDKTGSCGPVSSNGANGVKGFSFKYGNGVWIGRTKSISS RNGFEMIWDPNGWTGTDNNFSIKQDIVGINEWSGYSGSFVQHP TGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGSW PDGAELPFTIDKdykdddk**	38

N9/N1 Long Stalk TMD Chimera		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGATCCTGTGTACCTCCGCGACCGCTATC ACAATTGGGGCCATCGCCGTTCTGATCGGAATAGCAAACCTCGGA CTGAACATCGGACTTCATTTG<i>aagccaggatgtaattgcagtcattctcagccccgagacaacaacacgtcccaaaccattattaacaactactataacgaaacaaatatcactaacattcagatggaggagagacacatctcgtaat</i> <i>ttcaacaacctcacaaaaggactctgcacaGTG</i> AGCGGATGGGCCATCTATAGCAAAGATAATAGCATCCGAATCGGC TCAAAGGGGACGTATTTGTCATCCGCGAACCTTTATATCATGC TCACCACTTGAGTGTCGTACATTCTTCCTCACCCAGGGTGCGCTT CTGAATGACAAACACTCCAATGGCACTATAAAAGACCGATCCCC TATCGGACTCTGATGTCATGTCCAATTGGCGAAGTGCCTAGCCCG TATAATTCACGATTTGAATCAGTAGCTTGGAGCGCTTCTGCTTGC CATGATGGTATAAATTGGCTTACAATCGGAATTTCCGGCCCCGAT AATGGCGCTGTGGCGGTGCTCAAATACAATGGTATCATCACCGAT ACAATCAAGTCTTGGCGCAACAACATTCTCCGAACCCAGGAAAGT GAGTGTGCATGCGTAAATGGTAGCTGTTTCACCGTCATGACGGAC GGGCCTTCCGACGGCCAGGCATCATACAAAATCTTCCGCATCGAG AAAGGAAAGATCGTCAAAAGTGTTGAAATGAATGCTCCCAATTAC CATTATGAAGAATGCAGCTGTTATCCCGATTCTCTGAGATCACC TGCGTATGTAGGGATAATTGGCACGGGTCCAATCGGCCGTGGGT TCATTTAATCAGAACCTTGAGTATCAGATTGGTTACATCTGCTCT GGGATATTTGGTGATAATCCGCGACCAAATGACAAAACAGGGTCT TGCGGCCCAGTGAGTTCAAATGGCGCCAATGGTGTAAAAGGTTTC TCTTTCAAATACGGCAATGGCGTTTGGATTGGCAGGACCAAAAGT ATCAGCAGTAGGAATGGATTGAGATGATTTGGGATCCCAATGGT TGGACGGGGACTGATAATAATTTCTCCATTAAGCAAGATATCGTC GGCATTAAACGAATGGAGCGGTTACTCCGGCTCCTTTGTCCAACAT CCTGAACTGACCGGCCTCGATTGTATCCGTCCATGTTTCTGGGTA GAACTCATCAGGGGTAGACCTAAAGAGAATACCATTTGGACATCT GGTTCTAGCATCAGCTTTTGCGGTGTCAACTCAGATACAGTTGGA TGGTCTTGGCCTGACGGGGCAGAATTGCCTTTTACCATAGATAAG gattacaaggatgacgacgataagTAATAA	39
Amino Acid Sequence	MNPNQKILCTSATAITIGAIAVLIGIANLGLNIGLHL<i>kpgcncsh</i> <i>sqpetntnsqtiinnynnetnitniqmeertsrnfnnltkglctV</i> SGWAIYSKDNSIRIGSKGDVFVIREPFISCSPLECRTFFLTQAL LNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWSASAC HDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQES ECACVNGSCFTVMTDGPSDGQASYKIFRIEKGKIVKSVMENAPNY HYEECSYPDSSEITCVCRDNWHGNSNRPWVSFNQNLEYQIGYICS GIFGDNPRPNDKTGSCGPVSSNGANGVKGFSEFKYGNVWIGRTKS ISSRNGFEMIWDPNGTGTDNDFSQKQDIVGINEWSGYSGSFVQH PELTGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNSDTVG WSWPDGAELPFTIDKdykdddk**	40
N9/N1 Short Stalk Chimera		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGATCATCACGATTGGATCAGTTTGCATG ACAATCGGGATGGCAAATCTGATACTCCAAATTGGTAATATTATT AGTATTTGGATCaagccaggatgtaattgcagtcattctcagccc gagacaacaacacgtcccaaaccattattaacaactactataac gaaacaaatatcactaacattcagatggaggagagagaacatctAGC GTCAAACCTCGCTGGCAACTCAAGCCTCTGTCCAGTGAGCGGATGG GCCATCTATAGCAAAGATAATAGCATCCGAATCGGCTCAAAGGG	41

	GACGTATTTGTCATCCGCGAACCCTTTATATCATGCTCACCACCTT GAGTGTGCTACATTCTTCCTCACCCAGGGTGCGCTTCTGAATGAC AAACACTCCAATGGCACTATAAAAAGACCGATCCCCCTATCGGACT CTGATGTCATGTCCAATTGGCGAAGTGCCTAGCCCCGTATAATTCA CGATTTGAATCAGTAGCTTGGAGCGCTTCTGCTTGCCATGATGGT ATAAATTGGCTTACAATCGGAATTTCCGGCCCCGATAATGGCGCT GTGGCGGTGCTCAAATACAATGGTATCATCACCGATAACAATCAAG TCTTGGCGCAACAACATTCTCCGAACCCAGGAAAGTGAGTGTGCA TGCGTAAATGGTAGCTGTTTCACCGTCATGACGGACGGGCCTTCC GACGGCCAGGCATCATACAAAATCTTCCGCATCGAGAAAGGAAAG ATCGTCAAAAGTGTTGAAATGAATGCTCCCAATTACCATTATGAA GAATGCAGCTGTTATCCCGATTCTCTGAGATCACCTGCGTATGT AGGGATAATTGGCACGGGTCCAATCGGCCGTGGGTTTCATTTAAT CAGAACCTTGAGTATCAGATTGGTTACATCTGCTCTGGGATATTT GGTGATAATCCGCGACCAAATGACAAAACAGGGTCTTGCGGCCCA GTGAGTTCAAATGGCGCCAATGGTGTAAGGTTTCTCTTTCAA TACGGCAATGGCGTTTGGATTGGCAGGACCAAAAGTATCAGCAGT AGGAATGGATTTCGAGATGATTTGGGATCCCAATGGTTGGACGGGG ACTGATAATAATTTCTCCATTAAGCAAGATATCGTCGGCATTAAC GAATGGAGCGGTTACTCCGGCTCCTTTGTCCAACATCCTGAACCTG ACCGGCCTCGATTGTATCCGTCCATGTTTCTGGGTAGAACTCATC AGGGGTAGACCTAAAGAGAATACCATTGGACATCTGGTTCTAGC ATCAGCTTTTGCGGTGTCAACTCAGATACAGTTGGATGGTCTTGG CCTGACGGGGCAGAATTGCCTTTTACCATAGATAAGgattacaag gatgacgacgataagTAATAA	
Amino Acid Sequence	MNPNQKIITIGSVCMTIGMANLILQIGNIISIWIkpgcncshsqp ettnstsqtiinnyynetnitiqmeertsSVKLAGNSSLCPVSGW AIYSKDNSIRIGSKGDVVFIREPFISCSPLECRTFFLTQGALLND KHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWSASACHDG INWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQESECA CVNGSCFTVMTDGP SDGQASYKIFRIEKGIKIVKSVEMNAPNYHYE ECSCYPDSSEITCVCRDNWHGNSNRPWVSFNQNL EYQIGYICSGIF GDNPRPNDKTGSCGPVSSNGANGVKGFSFKYGNVWIGRTKSISS RNGFEMIWDPNGWGTGTDNNFSIKQDIVGINEWSGYSGSFVQHP ELTGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNSDTVGSW PDGAELPFTIDKdykdddk**	42
N9/N1 Short Stalk TMD Chimera		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGATCCTGTGTACCTCCGCGACCGCTATC ACAATTGGGGCCATCGCCGTTCTGATCGGAATAGCAAACCTCGGA CTGAACATCGGACTTCATTTGaagccaggatgtaattgcagtc atctcagccccgagacaacaaacacgtcccaaaccattattaacaac tactataacgaaacaaatatcactaacattcagatggaggagaga acatctAGCGTCAAACCTCGCTGGCAACTCAAGCCTCTGTCCAGTG AGCGGATGGGCCATCTATAGCAAAGATAATAGCATCCGAATCGGC TCAAAAGGGGACGTATTTGTCATCCGCGAACCCTTTATATCATGC TCACCACCTTGAGTGTCGTACATTCTTCCTCACCCAGGGTGCGCTT CTGAATGACAAACACTCCAATGGCACTATAAAAAGACCGATCCCC TATCGGACTCTGATGTCATGTCCAATTGGCGAAGTGCCTAGCCCG TATAATTCACGATTTGAATCAGTAGCTTGGAGCGCTTCTGCTTGC CATGATGGTATAAATTGGCTTACAATCGGAATTTCCGGCCCCGAT AATGGCGCTGTGGCGGTGCTCAAATACAATGGTATCATCACCGAT ACAATCAAGTCTTGGCGCAACAACATTCTCCGAACCCAGGAAAGT	43

	GAGTGTGCATGCGTAAATGGTAGCTGTTTCACCGTCATGACGGAC GGGCCTTCCGACGGCCAGGCATCATACAAAATCTTCCGCATCGAG AAAGGAAAGATCGTCAAAAGTGTTGAAATGAATGCTCCCAATTAC CATTATGAAGAATGCAGCTGTTATCCCGATTCTCTGAGATCACC TGCGTATGTAGGGATAATTGGCACGGGTCCAATCGGCCGTGGGTT TCATTTAATCAGAACCTTGAGTATCAGATTGGTTACATCTGCTCT GGGATATTTGGTGATAATCCGCGACCAAATGACAAAACAGGGTCT TGCGGGCCAGTGAGTTCAAATGGCGCCAATGGTGTAAAAGGTTTC TCTTTCAAATACGGCAATGGCGTTTGGATTGGCAGGACCAAAAGT ATCAGCAGTAGGAATGGATTTCGAGATGATTTGGGATCCCAATGGT TGGACGGGGACTGATAATAATTTCTCCATTAAGCAAGATATCGTC GGCATTAAACGAATGGAGCGGTTACTCCGGCTCCTTTGTCCAACAT CCTGAACGACCGGCCTCGATTGTATCCGTCCATGTTTCTGGGTA GAACTCATCAGGGGTAGACCTAAAGAGAATAACCATTGGACATCT GGTTCTAGCATCAGCTTTTGCGGTGTCAACTCAGATACAGTTGGA TGGTCTTGGCCTGACGGGGCAGAATTGCCTTTTACCATAGATAAG gattacaaggatgacgacgataagTAATAA	
Amino Acid Sequence	MNPNQKILCTSATAITIGAIAVLIGIANLGLNIGLHLkpgcncsh sqpetntntsqtinnyynetnitniqmeertsSVKLAGNSSSLCPV SGWAIYSKDNSIRIGSKGDVVFVIREPFISCSPLECRTFFLTQGal LNDKHSNGTIKDRSPYRTLMSCPIGEVPSPYNSRFESVAWSASAC HDGINWLTIGISGPDNGAVAVLKYNGIITDTIKSWRNNILRTQES ECACVNGSCFTVMTDGPSDGQASYKIFRIEKGIKIVKSVEMNAPNY HYEECSYCPDSSEITCVCRDNWHGSNRPWVSFNQNLEYQIGYICS GIFGDNPRPNDKTGSCGPVSSNGANGVKGFSEFKYGNVWIGRTKS ISSRNGFEMIWDPNWGTGTDNNFSIKQDIVGINEWSGYSGSFVQH PELTGLDCIRPCFWVELIRGRPKENTIWTSGSSISFCGVNSDVTG WSWPDGAELPFTIDKdykdddk* *	44
N9/N1 TMD Chimera		
Nucleic Acid Sequence	ATGAACCCAAATCAGAAGATCCTGTGTACCTCCGCGACCGCTATC ACAATTGGGGCCATCGCCGTTCTGATCGGAATAGCAAACCTCGGA CTGAACATCGGACTTCATTTGTCCCACAGCATCCAACTCGGCAAC CAGAACCAAATCGAAACGTGTAATCAATCTGTAATCACTTATGAA AATAACACGTGGGTGAATCAAACGTATGTGAATATCTCCAATACC AATTTTCGCTGCTGGGCAAAGTGTGGTAAGCGTCAAACTCGCTGGC AACTCAAGCCTCTGTCCAGTGAGCGGATGGGCCATCTATAGCAAA GATAATAGCATCCGAATCGGCTCAAAAGGGGACGTATTTGTCATC CGCGAACCCCTTTATATCATGCTCACCACTTGAGTGTCGTACATTC TTCCTCACCCAGGGTGCGCTTCTGAATGACAAACACTCCAATGGC ACTATAAAAGACCGATCCCCCTATCGGACTCTGATGTCATGTCCA ATTGGCGAAGTGCCTAGCCCGTATAATTCACGATTTGAATCAGTA GCTTGGAGCGCTTCTGCTTGCCATGATGGTATAAATTGGCTTACA ATCGGAATTTCCGGCCCCGATAATGGCGCTGTGGCGGTGCTCAA TACAATGGTATCATCACCGATAACAATCAAGTCTTGGCGCAACAAC ATTCTCCGAACCCAGGAAAGTGAGTGTGCATGCGTAAATGGTAGC TGTTTCACCGTCATGACGGACGGGCCTTCCGACGGCCAGGCATCA TACAAAATCTTCCGCATCGAGAAAGGAAAGATCGTCAAAGTGTT GAAATGAATGCTCCCAATTACCATTATGAAGAATGCAGCTGTTAT CCCGATTCTCTGAGATCACCTGCGTATGTAGGGATAATTGGCAC GGGTCCAATCGGCCGTGGGTTTTCATTTAATCAGAACCTTGAGTAT CAGATTGGTTACATCTGCTCTGGGATATTTGGTGATAATCCGCGA CCAAATGACAAAACAGGGTCTTGCGGCCAGTGAGTTCAAATGGC	45

	GCCAATGGTGTAAAAGGTTTCTCTTTCAAATACGGCAATGGCGTT TGGATTGGCAGGACCAAAAGTATCAGCAGTAGGAATGGATTTCGAG ATGATTTGGGATCCCAATGGTTGGACGGGGACTGATAATAATTTT TCCATTAAGCAAGATATCGTCGGCATTAAACGAATGGAGCGGTTAC TCCGGCTCCTTTGTCCAACATCCTGAACTGACCGGCCTCGATTGT ATCCGTCCATGTTTCTGGGTAGAACTCATCAGGGGTAGACCTAAA GAGAATACCATTGACATCTGGTTCTAGCATCAGCTTTTGCAGGT GTCAACTCAGATACAGTTGGATGGTCTTGGCCTGACGGGGCAGAA TTGCCTTTTACCATAGATAAG gattacaaggatgacgacgataag TAATAA	
Amino Acid Sequence	MNPNQK ILCTSATAITIGAIAVLIGIANLGLNIGLHLSHSIQ LG NQNIETCNQSVITYENNTWVNQTYVNI SNTNFAAGQSVSVKLAG NSSLCPVSGWAIYSKDNSIRIGSKGDV FVIREPFISCSPLECRTF FLTQGALLNDKHSNGTIKDRSPYRTL MSCPIGEVPSPYNSRFESV AWSASACHDGINWLTIGISGPDNGA VAVLKYNGIITDTIKSWRNN ILRTQESEACVNGSCFTVMTDGP SDGQASYKIFRIEKGKIVKSV EMNAPNYHYEECSCYPDSSEITC VCRDNWHGNSRNPWVSFNQ NLEYQIGYICSGIFGDNPRPNDKT GSCGPVSSNGANGVKGF SFKYGNVWIGRTKSISSRNGFEMI WDPNGWTGTDNNFSIKQDIVG INEWSGYSGSFVQHP ELTGLDCIRPCFWVELIRGRPK ENTIWTSGSSISFCG VNSDTVGSWPDGAELPFTIDK dykdddk **	46
Nucleic acid sequence encoding A/Yunnan/0129/2017 N9 TMD (SEQ ID NO: 28) and amino acid sequence SEQ ID NO: 28 is shown in uppercase bold italic. Nucleic acid sequence encoding short stalk domain (SEQ ID NO: 30) and the amino acid sequence of SEQ ID NO: 30 shown in lowercase. Nucleic acid sequence encoding long stalk domain (SEQ ID NO: 31) and the amino acid sequence of SEQ ID NO: 31 shown in lowercase italic. Nucleic acid sequence encoding FLAG-tag (SEQ ID NO: 8) and amino acid sequence of FLAG-tag (SEQ ID NO: 8) shown in bold lowercase. TAATAA (SEQ ID NO: 47) in nucleic acid sequence encodes two stop codons; ** in amino acid sequence indicates stop codons.		

[0052] In some embodiments, the chimeric influenza A virus neuraminidase protein is encoded by a portion of the nucleic acid sequences identified in Table 4 above (*i.e.*, SEQ ID NO: 33, SEQ ID NO: 35, SEQ ID NO: 37, SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43, or SEQ ID NO: 45). For example, the chimeric influenza A virus neuraminidase protein may be encoded by a portion of the nucleic acid sequences identified in Table 4 above lacking the sequence encoding the amino acid sequence of SEQ ID NO: 8 (FLAG-tag) and/or SEQ ID NO: 47 (double stop codon).

[0053] In some embodiments, the portion of the nucleic acid sequences identified in Table 4 above encodes a portion of the amino acid sequences identified in Table 4 above (*i.e.*, SEQ ID NO: 34, SEQ ID NO: 36, SEQ ID NO: 38, SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44, or SEQ ID NO: 46). For example, the chimeric influenza A virus neuraminidase

protein may comprise a portion of the amino acid sequences identified in Table 4 lacking the amino acid sequence of SEQ ID NO: 8 (FLAG-tag).

[0054] In some embodiments, the chimeric influenza A virus neuraminidase protein is encoded by nucleic acids 1-1416 of SEQ ID NO: 45. In accordance with such embodiments, nucleic acids 1-1416 encode for amino acids 1-472 of SEQ ID NO: 46.

Vector Constructs

[0055] Nucleic acid sequences encoding a chimeric influenza A virus neuraminidase protein of the present disclosure may be inserted into a vector capable of replicating and expressing the nucleic acid molecules in host cells. Accordingly, another aspect of the present disclosure relates to a vector encoding a chimeric influenza A virus neuraminidase protein, where the vector comprises the nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein according to the present disclosure.

[0056] The term “vector” refers to any nucleic acid construct capable of directing the expression of one or more sequences of interest (*e.g.*, a chimeric influenza A virus neuraminidase protein according to the present disclosure) in a host cell. Thus, the term includes cloning and expression vehicles, as well as viral vectors. The term is used interchangeable with the terms “nucleic acid expression vector” and “expression cassette.”

[0057] In some embodiments, the vector further comprises: a promoter and a termination sequence, where the promoter and termination sequence are operably coupled to the nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein.

[0058] The terms “operably coupled” and “operably linked” are used interchangeably and refer to an arrangement of elements wherein the components so described are configured so as to perform their usual function. Thus, a given promoter operably coupled to a coding sequence is capable of effecting the expression of the coding sequence when active. The promoter need not be contiguous with the coding sequence, so long as it functions to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between the promoter sequence and the coding sequence and the promoter sequence can still be considered “operably coupled” to the coding sequence.

[0059] Many vectors are available in the art and can be used. Each vector may contain various components that may be adjusted and optimized for compatibility with the particular host cell. As noted above, the vector components may include, but are not limited to, a promoter, a nucleic acid sequence encoding protein of interest, and a transcription termination sequence. Suitable additional vector components include, without limitation, an origin of replication, a

selection marker gene, a signal sequence, an enhancer, a cloning site, a polyadenylation site, and an affinity tag sequence.

[0060] Promoters are positioned 5' (upstream) to the genes that they control. Many eukaryotic promoters contain two types of recognition sequences: TATA box and the upstream promoter elements. The TATA box, located 25-30 bp upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase II to begin RNA synthesis at the correct site. In contrast, the upstream promoter elements determine the rate at which transcription is initiated. These elements can act regardless of their orientation, but they must be located within 100 to 200 bp upstream of the TATA box. Suitable exemplary promoters include a CMV promoter (*e.g.*, CMV immediate early promoter), an EF-1 alpha promoter, an SV40 early promoter, a chicken beta-actin promoter, HIV-LTR, and adenovirus major late promoter (Ad MLP).

[0061] Enhancer elements can stimulate transcription up to 1000-fold from linked homologous or heterologous promoters. Enhancer elements often remain active even if their orientation is reversed (Li et al., "High Level Desmin Expression Depends on a Muscle-Specific Enhancer," *J. Bio. Chem.* 266(10):6562-6570 (1991), which is hereby incorporated by reference in its entirety). Furthermore, unlike promoter elements, enhancers can be active when placed downstream from the transcription initiation site, *e.g.*, within an intron, or even at a considerable distance from the promoter (Yutzey et al., "An Internal Regulatory Element Controls Troponin I Gene Expression," *Mol. Cell. Bio.* 9(4):1397-1405 (1989), which is hereby incorporated by reference in its entirety). Suitable enhancer elements include, without limitation, the SV40 early gene enhancer, the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, and elements derived from human CMV.

[0062] Suitable affinity tag sequences are well known in the art (*see, e.g.*, Kimple et al., "Overview of Affinity Tags for Protein Purification," *Curr. Protoc. Protein Sci.* 73:Unit 9.9 (2013), which is hereby incorporated by reference in its entirety). Exemplary affinity tag sequences include, without limitation, a FLAG tag, an E2 epitope tag, a histidine affinity tag, an HSV epitope tag, a KT3 epitope tag, a Myc epitope tag, a polyaspartate tag, a polycysteine tag, a polyhistidine tag, a polyphenylalanine tag, an S1 tag, an S tag, a Strep tag, a VSV-G tag, an AU epitope tag, and an AU5 epitope tag, an HA tag, and an AU1 tag (*see, e.g.*, Table 5).

Table 5. Exemplary Affinity Tags

Description	Amino Acid Sequence	SEQ ID NO:
FLAG tag	DYKDDDDK	8
E2 epitope tag	SSTSSDFRDR	9

Histidine affinity tag (HAT)	KDHLIHNVHKEFHAHAHNK	10
HSV epitope tag	QPELAPED	11
KT3 epitope tag	KPPTPPPEPET	12
Myc epitope tag	CEQKLISEEDL	13
Polyaspartate tag	DDDDD	14
Polycysteine tag	CCCC	15
Polyhistidine tag	HHHHHH	16
Polyphenylalanine tag	FFFFFFFFFFFF	17
S1 tag	NANNPDWDF	18
S tag	KETAAAKFERQHMD	19
Strep-tag	WSHPQFEK	20
Strep-tag	AWAHPQPGG	21
VSV-G	YTDIEMNRLGK	22
AU1 epitope tag	DTYRYI	23
AU5 epitope tag	TDFYLK	24

[0063] In some embodiments, the vector is an expression vector. For example, the expression vector may be a mammalian expression vector such as a pCAGGS expression vector (Niwa et al., “Efficient Selection for High-Expression Transfectants with a Novel Eukaryotic Vector,” *Gene* 108:193-200 (1991), which is hereby incorporated by reference in its entirety) or a pCDNA 3.1 vector. In some embodiments, the vector is a pCAGGS vector comprising a sequence of SEQ ID NO: 32, as follows:

ACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTC
CGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCA
TTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCATTGACGT
CAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATG
CCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCAG
TACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATT
ACCATGGTCGAGGTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCCTCCCCA
CCCCCAATTTTGTATTTATTTATTTTAAATTATTTTGTGCAGCGATGGGGGCGGGGGG
GGGGGGGGGCGCGGCCAGGCGGGGCGGGGCGGGGCGAGGGGCGGGGCGGGGCGAGGCGG
AGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCGCTCCGAAAGTTTCCTTTTATGGCGAGG
CGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCGCGCGGCGGGGCGGGGAGTCGCTGCGAC
GCTGCCTTCGCCCCGTGCCCCGCTCCGCGCCGCTCGCGCCGCCCCGCCCCGGCTCTGAC
TGACCGCGTTACTCCACAGGTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATT
AGCGCTTGGTTTAAATGACGGCTTGTTCCTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGC
TCCGGGAGGGCCCTTTGTGCGGGGGAGCGGCTCGGGGGGTGCGTGCGTGTGTGTGTGCG
TGGGGAGCGCGCGTGCAGCTCCGCGCTGCCCGGCGGCTGTGAGCGCTGCGGGCGCGGCG
CGGGGCTTTGTGCGCTCCGCAGTGTGCGGAGGGGAGCGCGGCCGGGGGCGGTGCCCCGC
GGTGCGGGGGGGGCTGCGAGGGGAACAAAGGCTGCGTGCGGGGTGTGTGCGTGCGGGGGT

GAGCAGGGGGTGTGGGCGCGTCGGTCGGGCTGCAACCCCCCTGCACCCCCCTCCCCGAG
TTGCTGAGCACGGCCCGGCTTCGGGTGCGGGGCTCCGTACGGGGCGTGGCGCGGGGCTCG
CCGTGCCGGGCGGGGGGTGGCGGCAGGTGGGGGTGCCGGGCGGGGCGGGGCCGCCTCGGG
CCGGGGAGGGCTCGGGGGAGGGGCGCGGCGGCCCCCGGAGCGCCGGCGGGCTGTTCGAGGCG
5 CGGCGAGCCGCAGCCATTGCCTTTTATGGTAATCGTGCGAGAGGGCGCAGGGACTTCCTT
TGTCCCAAATCTGTGCGGAGCCGAAATCTGGGAGGCGCCGCCGCACCCCCTCTAGCGGGC
GCGGGGCGAAGCGGTGCGGCGCCGGCAGGAAGGAAATGGGCGGGGAGGGCCTTCGTGCGT
CGCCGCGCCGCGTCCCCTTCTCCCTCTCCAGCCTCGGGGCTGTCCGCGGGGGGACGGCT
GCCTTCGGGGGGGACGGGGCAGGGCGGGGTTCGGCTTCTGGCGTGTGACCGGCGGGCTCTA
10 GAGCCTCTGCTAACCATTGTCATGCCTTCTTCTTTTTCTACAGCTCCTGGGCAACGTGC
TGTTTATTGTGCTGTCTCATCATTTTGGCAAAGAATTTCGAGCTCATCGATGCATGGTACC
CGGGCATGCTCGAGATGAACACGATTAACATCGCTAAGAACGACTTCTCTGACATCGAAC
TGGCTGCTATCCCGTTCAACACTCTGGCTGACCATTACGGTGAGCGTTTAGCTCGCGAAC
AGTTGGCCCTTGAGCATGAGTCTTACGAGATGGGTGAAGCACGCTTCCGCAAGATGTTTG
15 AGCGTCAACTTAAAGCTGGTGAGGTGCGGATAACGCTGCCGCCAAGCCTCTCATCTACTA
CCCTACTCCCTAAGATGATTGCACGCATCAACGACTGGTTTGAGGAAGTGAAAGCTAAGC
GCGGCAAGCGCCCGACAGCCTTCCAGTTCTTCAAGAAATCAAGCCGGAAGCCGTAGCGT
ACATCACCATTAAAGACCACTCTGGCTTGCCTAACCAAGTGCTGACAATAACAACCGTTCAGG
CTGTAGCAAGCGCAATCGGTGCGGCCATTGAGGACGAGGCTCGCTTCGGTTCGTATCCGTG
20 ACCTTGAAGCTAAGCACTTCAAGAAAAACGTTGAGGAACAACTCAACAAGCGCGTAGGGC
ACGTCTACAAGAAAGCATTTATGCAAGTTGTGAGGCTGACATGCTCTCTAAGGGTCTAC
TCGGTGGCGAGGCGTGGTCTTCGTGGCATAAGGAAGACTCTATTTCATGTAGGAGTACGCT
GCATCGAGATGCTCATTGAGTCAACCGGAATGGTTAGCTTACACCGCCAAAATGCTGGCG
TAGTAGGTCAAGACTCTGAGACTATCGAACTCGCACCTGAATACGCTGAGGCTATCGCAA
25 CCCGTGCAGGTGCGCTGGCTGGCATCTCTCCGATGTTCCAACCTTGCGTAGTTCCTCCTA
AGCCGTGGACTGGCATTACTGGTGGTGGCTATTGGGCTAACGGTCGTGCTCCTCTGGCGC
TGGTGCGTACTCACAGTAAGAAAGCACTGATGCGCTACGAAGACGTTTACATGCCTGAGG
TGTACAAAGCGATTAAACATTGCGCAAAACACCGCATGGAAAATCAACAAGAAAGTCCTAG
CGGTGCGCAACGTAATCACCAAGTGGAAGCATTGTCCGGTCGAGGACATCCCTGCGATTG
30 AGCGTGAAGAACTCCCGATGAAACCGGAAGACATCGACATGAATCCTGAGGCTCTCACCG
CGTGGAACGTGCTGCCGCTGCTGTGTACCGCAAGGACAAGGCTCGCAAGTCTCGCCGTA
TCAGCCTTGAGTTCATGCTTGAGCAAGCCAATAAGTTTGCTAACCATTAAGGCCATCTGGT
TCCCTTACAACATGGACTGGCGCGGTGCTGTTTACGCTGTGTCAATGTTCAACCCGCAAG
GTAACGATATGACCAAAGGACTGCTTACGCTGGCGAAAGGTAAACCAATCGGTAAGGAAG
35 GTTACTACTGGCTGAAAATCCACGGTGCAAACGTGTGCGGGTGTGCGATAAGGTTCCGTTCC

CTGAGCGCATCAAGTTCATTGAGGAAAACACGAGAACATCATGGCTTGCGCTAAGTCTC
CACTGGAGAACACTTGGTGGGCTGAGCAAGATTCTCCGTTCTGCTTCCTTGCGTTCTGCT
TTGAGTACGCTGGGGTACAGCACCACGGCCTGAGCTATAACTGCTCCCTTCCGCTGGCGT
TTGACGGGTCTTGCTCTGGCATCCAGCACTTCTCCGCGATGCTCCGAGATGAGGTAGGTG
5 GTCGCGCGGTAACTTGCTTCCTAGTGAAACCGTTCAGGACATCTACGGGATTGTTGCTA
AGAAAGTCAACGAGATTCTACAAGCAGACGCAATCAATGGGACCGATAACGAAGTAGTTA
CCGTGACCGATGAGAACACTGGTGAAATCTCTGAGAAAGTCAAGCTGGGCACTAAGGCAC
TGGCTGGTCAATGGCTGGCTTACGGTGTTACTCGCAGTGTGACTAAGCGTTCAGTCATGA
CGCTGGCTTACGGGTCCAAAGAGTTCGGCTTCCGTCAACAAGTGCTGGAAGATACCATTC
10 AGCCAGCTATTGATTCCGGCAAGGGTCTGATGTTCACTCAGCCGAATCAGGCTGCTGGAT
ACATGGCTAAGCTGATTTGGGAATCTGTGAGCGTGACGGTGGTAGCTGCGGTTGAAGCAA
TGAAGTGGCTTAAAGTCTGCTGCTAAGCTGCTGGCTGCTGAGGTCAAAGATAAGAAGACTG
GAGAGATTCTTCGCAAGCGTTGCGCTGTGCATTGGGTAACTCCTGATGGTTTTCCCTGTGT
GGCAGGAATACAAGAAGCCTATTCAGACGCGCTTGAACCTGATGTTCCCTCGGTCAGTTCC
15 GCTTACAGCCTACCATTAAACACCAACAAAGATAGCGAGATTGATGCACACAAACAGGAGT
CTGGTATCGCTCCTAACTTTGTACACAGCCAAGACGGTAGCCACCTTCGTAAGACTGTAG
TGTGGGCACACGAGAAGTACGGAATCGAATCTTTTGCACTGATTCACGACTCCTTCGGTA
CCATTCCGGCTGACGCTGCGAACCTGTTCAAAGCAGTGCGCGAAACTATGGTTGACACAT
ATGAGTCTTGTGATGTACTGGCTGATTTCTACGACCAGTTCGCTGACCAGTTGCACGAGT
20 CTCAATTGGACAAAATGCCAGCACTTCCGGCTAAAGGTAACCTGAACCTCCGTGACATCT
TAGAGTCGGACTTCGCGTTTCGCGTAACGCCAAATCAATACGACTCCGGATCTTTTTCCCT
CTGCCAAAATTTATGGGGACATCATGAAGCCCCTTGAGCATCTGACTTCTGGCTAATAAA
GGAAATTTATTTTCATTGCAATAGTGTGTTGGAATTTTTTGTGTCTCTCACTCGGAAGGA
CATATGGGAGGGCAAATCATTTAAAACATCAGAATGAGTATTTGGTTTAGAGTTTGGCAA
25 CATATGCCCATATGCTGGCTGCCATGAACAAAGGTTGGCTATAAAGAGGTCATCAGTATA
TGAAACAGCCCCCTGCTGTCCATTCCCTTATTCATAGAAAAGCCTTGACTTGAGGTTAGA
TTTTTTTTATATTTTGTGTTTGTGTTATTTTTTTCTTTAACATCCCTAAAATTTTCCTTAC
ATGTTTTACTAGCCAGATTTTTTCCTCCTCTCCTGACTACTCCAGTCATAGCTGTCCCTC
TTCTCTTATGGAGATCCCTCGACCTGCAGCCCAAGCTTGCGGTAATCATGGTCATAGCTG
30 TTTCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATA
AAGTGTAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCA
CTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCGGATCCGCATCTCAATTAGTCAG
CAACCATAGTCCCGCCCCCTAACTCCGCCCCATCCCGCCCCCTAACTCCGCCCAGTTCCGCCC
ATTCTCCGCCCCATGGCTGACTAATTTTTTTTTATTTATGCAGAGGCCGAGGCCGCTCGG
35 CCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAA

AGCTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTT
CACAAATAAAGCATTTTTTTTCTACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGT
ATCTTATCATGTCTGGATCCGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGT
TTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCTGTTTCGG
5 CTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGG
GATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAG
GCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCTGACGAGCATCACAAAAATCGA
CGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCT
GGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGTTACCGGATACCTGTCCGCC
10 TTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTTCG
GTGTAGGTCGTTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGC
TGCGCCTTATCCGGTAACATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCA
CTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCT
15 CTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACC
ACCGCTGGTAGCGGTGGTTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAGGA
TCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACCTCA
CGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAAT
TAAAAATGAAGTTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTAC
20 CAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTTCGTTTCATCCATAGTT
GCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGT
GCTGCAATGATACCGCGAGACCCACGCTCACC GGCTCCAGATTTATCAGCAATAAACCAG
CCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCT
ATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTT
25 GTTGCCATTGCTACAGGCATCGTGGTGTACGCTCGTCGTTTGGTATGGCTTCATTCAGC
TCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTT
AGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGAGTGTTATCACTCATG
GTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTG
ACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCT
30 TGCCCGGCGTCAATACGGGATAATAACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATC
ATTGGAAAACGTTCTTCGGGGCGAAAACCTCTCAAGGATCTTACCGCTGTTGAGATCCAGT
TCGATGTAACCCACTCGTGACCCCACTGATCTTCAGCATCTTTTACTTTTACCAGCGTT
TCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGG
AAATGTTGAATACTCATACTCTTCCTTTTTTCAATATTATTGAAGCATTTATCAGGGTTAT

TGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCG
CGCACATTTCCCCGAAAAGTGCCACCTGGGTGACATTGATTATTG (SEQ ID NO: 32)

Host Cells

[0064] Another aspect of the present disclosure relates to a host cell comprising a nucleic acid molecule according to the present disclosure. In some embodiments, the host cell is a eukaryotic host cell. For example, the host cell may be a mammalian cell.

[0065] Suitable mammalian cells include, but are not limited to, human embryonic kidney (HEK) cells (*e.g.*, HEK293 cells, HEK 293F cells), Chinese hamster ovary (CHO) cells (*e.g.*, CHO Toronto/CHO pro 3, CHO K1, DG 44, DXB11, CHO K1 SV, CHOK1SV GS-KO), HeLa cells, COS cells, PC3 cells, African Green Monkey kidney cells (*e.g.*, Vero cells), baby hamster kidney (BHK) cells (*e.g.*, BHK21), PER.C6[®] cells, and Madin-Darby canine kidney (MDCK) cells (*e.g.*, MDCK cells, MDCK-HA cells, MDCK/London cells, MDCK.2 cells, MDCK SIAT-1 cells).

[0066] Different host cells have characteristic and specific mechanisms for the posttranslational processing and modification of protein products. Appropriate cell lines or host systems may be chosen to ensure the correct modification and processing of the expressed chimeric influenza A virus neuraminidase protein(s). The above-described expression vectors may be introduced into appropriate host cells using conventional techniques in the art, *e.g.*, transformation, transfection, electroporation, calcium phosphate precipitation, and direct microinjection.

[0067] Once the vectors are introduced into host cells for protein production, host cells are cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. Methods for expression of therapeutic proteins are known in the art, see, *e.g.*, Paulina Balbas, Argelia Lorence (eds.) *Recombinant Gene Expression: Reviews and Protocols* (Methods in Molecular Biology), Humana Press; 2nd ed. 2004 (July 20, 2004) and Vladimir Voynov and Justin A. Caravella (eds.) *Therapeutic Proteins: Methods and Protocols* (Methods in Molecular Biology) Humana Press; 2nd ed. 2012 (June 28, 2012), which are hereby incorporated by reference in their entirety.

Virus Like Particles

[0068] As noted *supra*, neuraminidase can form virus-like particles (VLPs) following transfection of plasmid DNA into cell culture, independent of other viral proteins (Yondola et al., "Budding Capability of the Influenza Virus Neuraminidase can be Modulated by Tetherin," *J. Virol.* 85(6):2480-2491 (2011), which is hereby incorporated by reference in its entirety).

Accordingly, the present disclosure also relates to virus-like particles (VLPs) produced by host cells according to the present disclosure, as well as virus-like particle(s) comprising a chimeric influenza A virus neuraminidase protein according to the present disclosure. Suitable chimeric influenza A virus neuraminidase proteins are described in detail *supra*.

5 **[0069]** The terms “virus-like particle” or “VLP” are used interchangeably and refer to a nonreplicating, viral shell. VLPs are generally composed of one or more viral proteins, such as, but not limited to those proteins referred to as surface and/or envelope proteins, or particle-forming polypeptides derived from these proteins.

10 **[0070]** The presence of VLPs following recombinant expression of viral proteins (*e.g.*, chimeric influenza A virus neuraminidase proteins) can be detected using conventional techniques known in the art, such as by electron microscopy, biophysical characterization, and the like (*see, e.g.*, Baker et al., “Structures of Bovine and Human Papillomaviruses. Analysis by Cryoelectron Microscopy and Three-Dimensional Image Reconstruction,” *Biophys. J.* 60:1445-1456 (1991); and Hagensee et al., “Three-Dimensional Structure of Vaccinia Virus-Produced

15 Human Papillomavirus Type 1 Capsids,” *J. Virol.* 68:4503-4505 (1994), which are hereby incorporated by reference in their entirety). For example, VLPs can be isolated by density gradient centrifugation (*e.g.*, sucrose density gradient centrifugation) and/or identified by characteristic density banding. Additional methods of VLP purification include but are not limited to chromatographic techniques such as affinity, ion exchange, size exclusion, and reverse

20 phase procedures.

[0071] In some embodiments, the VLP comprises a single viral surface protein (*e.g.*, only influenza A virus neuraminidase proteins). In accordance with such embodiments, the VLP comprises chimeric influenza A virus neuraminidase proteins according to the present disclosure.

Methods of Producing Virus Like Particles (VLPs)

25 **[0072]** Another aspect of the present disclosure relates to a method of producing a virus-like particle comprising a chimeric influenza A virus neuraminidase protein. This method involves providing the vector according to the present disclosure; transfecting a host cell with the vector; and culturing the host cell in a cell culture comprising a supernatant under conditions effective to express the chimeric influenza A virus neuraminidase protein and allow budding of

30 virus-like particles comprising tetramers of the chimeric influenza A virus neuraminidase proteins into the supernatant.

[0073] Another aspect of the disclosure relates to a method of producing a virus-like particle comprising a chimeric influenza A virus neuraminidase protein. This method involves providing the host cell according to the present disclosure; and culturing the host cell in a cell

culture comprising a supernatant under conditions effective to express the chimeric influenza A virus neuraminidase protein and to allow budding of virus-like particles comprising tetramers of the chimeric influenza A virus neuraminidase proteins into the supernatant.

[0074] Suitable vectors, host cells, and chimeric influenza A virus neuraminidase

5 proteins according to the present disclosure are described in more detail *supra*.

[0075] In some embodiments, the host cell is a mammalian host cell. For example, the mammalian host cell may be selected from the group consisting of HEK293 cells, CHO cells, HeLa cells, Vero cells, BHK cells, and MDCK cells.

[0076] Host cells used to produce virus-like particles comprising chimeric influenza A

10 virus neuraminidase proteins as disclosed herein may be grown in media known in the art and suitable for culturing of the selected host cells. Examples of suitable media for mammalian host cells include Dulbecco's Modified Eagle's Medium (DMEM), Minimal Essential Medium (MEM), Expi293™ Expression Medium, and RPMI-1640.

[0077] In some embodiments, host cells are cultured in supplemented medium. For

15 example, cell culture medium may be supplemented with bovine serum albumin (BSA), fetal calf serum (FCS; also known as bovine calf serum), iron, and/or antibiotics (*e.g.*, penicillin, streptomycin). In some embodiments, host cells are cultured in DMEM with antibiotics (penicillin and streptomycin) and 10% iron supplemented bovine calf serum (ISBCS).

[0078] Host cells are cultured at suitable temperatures, such as from about 20°C to about

20 39°C, *e.g.*, from about 25°C to about 37°C, and CO₂ levels, such as 5% to 10%. In some embodiments, host cells are cultured at about 37°C and/or at CO₂ levels of about 5%. The pH of the medium is may be from about 6.8 to 7.4, *e.g.*, 7.0, depending mainly on the host organism.

[0079] The methods of producing VLPs according to the present disclosure may further

involve recovering the virus-like particles from the supernatant. Methods of recovering VLPs

25 from a supernatant are well known in the art (*see, e.g.*, Buffin et al., "Influenza A and B Virus-Like Particles Produced in Mammalian Cells are Highly Immunogenic and Induce Functional Antibodies," *Vaccine* 37(46):6857-6867 (2019) and Wu et al., "Mammalian Expression of Virus-Like Particles for Advanced Mimicry of Authentic Influenza Virus," *PLoS One* 5(3):e9784

(2010), which are hereby incorporated by reference in their entirety). In some embodiments, the

30 recovering step involves centrifugation, filtration, chromatography, and/or dialysis.

[0080] In some embodiments, the recovering step involves collecting the supernatant containing the virus-like particles and centrifuging the collected supernatant to pellet the virus-like particles.

Pharmaceutical Compositions

[0081] Another aspect of the present disclosure relates to a pharmaceutical composition comprising a plurality of virus-like particles according to the present disclosure and a pharmaceutically acceptable carrier.

5 [0082] The term “pharmaceutically acceptable carrier” refers to a carrier that does not cause an allergic reaction or other untoward effect in patients to whom it is administered and are compatible with the other ingredients in the formulation. Pharmaceutically acceptable carriers include, for example, pharmaceutical diluents, excipients, or carriers suitably selected with respect to the intended form of administration, and consistent with conventional pharmaceutical
10 practices. For example, solid carriers/diluents include, but are not limited to, a gum, a starch (*e.g.*, corn starch, pregelatinized starch), a sugar (*e.g.*, lactose, mannitol, sucrose, dextrose), a cellulosic material (*e.g.*, microcrystalline cellulose), an acrylate (*e.g.*, polymethylacrylate), calcium carbonate, magnesium oxide, talc, or mixtures thereof. Pharmaceutically acceptable carriers may further comprise minor amounts of auxiliary substances such as wetting or
15 emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the nucleic acid molecule described herein.

[0083] In certain circumstances, it will be desirable to deliver the disclosed immunogenic compositions in suitably-formulated pharmaceutical vehicles by one or more standard delivery routes, including, for example, subcutaneously, parenterally, intravenously, intramuscularly,
20 orally, intraperitoneally, transdermally, topically, by oral or nasal inhalation, or by direct injection to one or more cells, tissues, or organs.

[0084] For administration of an injectable aqueous solution, for example, the solution may be suitably buffered, if necessary, and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for
25 intravenous, intramuscular, and subcutaneous administration. A sterile aqueous medium that can be employed will be known to those of ordinary skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 mL of isotonic NaCl solution, and either added to 1000 mL of hypodermoclysis fluid or injected at the proposed site of infusion (*see, e.g.*, “Remington's Pharmaceutical Sciences” 15th Edition, pp. 1035-1038 and 1570-1580, which is
30 hereby incorporate by reference in its entirety). For human administration, pharmaceutical compositions should meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.

[0085] Sterile injectable compositions may be prepared by incorporating the disclosed pharmaceutical compositions in the required amount in the appropriate solvent with several of
35 the other ingredients enumerated above, as required, followed by filtered sterilization.

Generally, dispersions can be prepared by incorporating the selected sterilized active ingredient(s) into a sterile vehicle that contains the basic dispersion medium and the required other ingredients from those enumerated above. The compositions disclosed herein may also be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein), and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine, and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation, and in such amount as is effective for the intended application. The formulations are readily administered in a variety of dosage forms such as injectable solutions, topical preparations, oral formulations, including sustain-release capsules, hydrogels, colloids, viscous gels, transdermal reagents, intranasal and inhalation formulations, and the like.

[0086] The pharmaceutical compositions disclosed herein are not in any way limited to use only in humans, or even to primates, or mammals. In preferred embodiments, however, the compositions of the present disclosure may be formulated for administration to a mammal, including a human, for one or more therapeutic and/or prophylactic regimens. The disclosed pharmaceutical compositions may also be formulated for veterinary administration, including, for example, to selected livestock, exotic or domesticated animals, companion animals (including pets and such like), non-human primates, as well as zoological or otherwise captive specimens, and such like.

[0087] In some embodiments, the composition is lyophilized. For example, the composition may be lyophilized in a sterile container.

[0088] As used herein, the term “sterile” refers to a system or components of a system free of infectious agents, including, but not limited to, bacteria, viruses, and bioactive RNA or DNA.

Methods of Eliciting an Immune Response

[0089] Another aspect of the present disclosure relates to a method of eliciting an immune response in a subject. This method involves administering an effective amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure, where said administering is effective to elicit an immune response in the subject.

[0090] The term “immune response” refers to the development in a subject of a humoral and/or a cellular immune response to an antigen present in, *e.g.*, a pharmaceutical composition of interest (*e.g.*, a pharmaceutical composition comprising an effective amount of a chimeric influenza A virus neuraminidase protein, a virus like particle comprising a chimeric influenza A virus neuraminidase protein, or a chimeric influenza A virus neuraminidase protein).

[0091] The term “humoral immune response” refers to an immune response mediated by antibody molecules, while a “cellular immune response” is one mediated by T-lymphocytes and/or other white blood cells. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells (CTLs). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. The term “cellular immune response” also refers to the production of cytokines, chemokines, and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4⁺ and CD8⁺ T-cells. Hence, an immunological response may include one or more of the following effects: the production of antibodies by B-cells; and/or the activation of suppressor T-cells and/or $\gamma\Delta$ T-cells directed specifically to an antigen or antigens present in the composition or vaccine of interest. These responses may serve to neutralize infectivity, and/or mediate antibody-complement, or antibody dependent cell cytotoxicity (ADCC) to provide protection to an immunized host. Such responses can be determined using standard immunoassays and neutralization assays, which are well known in the art.

[0092] Thus, administering an effective amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle, or pharmaceutical composition according to the present disclosure is effective to induce a humoral immune response and/or a cellular immune response. In some embodiments, the immune response is a humoral immune response. In other embodiments, the immune response is a cellular immune response.

[0093] The induced immune response may be effective to reduce the risk of infection or prevent infection with an influenza virus.

[0094] Suitable subjects include, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including

domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like. In some embodiments, the subject is a mammal (*e.g.*, a human). The term does not denote a particular age. Thus, suitable subjects include newborn, infant, adolescent, adult, and geriatric individuals.

5 **[0095]** An “effective dose” generally refers to that amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure sufficient to induce immunity, to prevent and/or ameliorate an infection or to reduce at least one symptom of an infection and/or to enhance the efficacy of another dose of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle
10 (VLP), or pharmaceutical composition according to the present disclosure. An effective dose may refer to the amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure sufficient to delay or minimize the onset of an infection. An effective dose may also refer to the amount of VLPs that provides a therapeutic benefit in the treatment or management of an infection.
15 Further, an effective dose is the amount with respect to the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of an infection. An effective dose may also be the amount sufficient to enhance a subject's own immune response against a subsequent
20 exposure to an infectious agent. Levels of immunity can be monitored, *e.g.*, by measuring amounts of neutralizing secretory and/or serum antibodies, *e.g.*, by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay. In the case of an immunogenic composition, an effective dose is one that induces an immune response in a subject. In some embodiments, the immune response is sufficient to prevent disease and/or
25 reduce the severity of symptoms of infection with an influenza A virus.

[0096] In some embodiments, the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure is administered at a dose of 1-50 micrograms of total NA protein incorporated into VLPs. For example, the compositions of the present disclosure may be administered at a dose of
30 1-50 micrograms, 1-40 micrograms, 1-30 micrograms, 1-20 micrograms, 1-10 micrograms, 10-50 micrograms, 20-50 micrograms, 30-50 micrograms, 40-50 micrograms, 10-40 micrograms, or 20-30 micrograms of total NA protein incorporated into VLPs.

[0097] As “effective amount” refers to an amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according
35 to the present disclosure necessary or sufficient to realize a desired biologic effect. An effective

amount of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure would be the amount that achieves a selected result, and such an amount could be determined as a matter of routine experimentation by a person skilled in the art. For example, an effective amount for preventing, treating and/or ameliorating an infection could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen specific immune response upon exposure to the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure. The term is also synonymous with “sufficient amount.”

[0098] In some embodiments, administering the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure is sufficient induce an immune response against antigenic determinants of the first influenza A virus protein. For example, administering may be effective to induce an immune response against antigenic determinants of the head domain of the first influenza A virus protein.

[0099] The chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure may be administered to a subject by any mode of delivery including, without limitation, subcutaneously, parenterally, intravenously, intramuscularly, orally, intraperitoneally, transdermally, topically, by oral or nasal inhalation, or by direct injection to one or more cells, tissues, or organs. In some embodiments, the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure is administered intramuscularly.

[0100] The chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure may be administered as a single dose or in multiple doses (*e.g.*, two doses, three doses, four doses, five doses, six doses, seven doses, eight doses, nine doses, or more).

[0101] In some embodiments, the compositions of the present disclosure are administered in one, two, or three doses. In accordance with such examples, the timing between the doses can be at least 1 week, at least 2 weeks, at least 3 weeks, at least 4 weeks, at least 6 weeks, at least 8 weeks, at least 12 weeks, at least 2 months, at least 3 months, at least 4 months, at least 5 months, at least 6 months, at least 1 year, at least 2 years, or at least 5 years, such as 1-4 weeks, 2-3 weeks, 1-6 months, 2-4 months, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, 8 weeks, 9 weeks, 10 weeks, 12 weeks, 1 month, 2 months, 3, months, 4, months, 5 months, 6 months, 1 year, 2 years, 5 years, or 10 years, or combinations thereof (such

as where there are at least three administrations, wherein the timing between the first and second, and second and third doses, can be the same or different).

[0102] The dose administered to the subject can depend on the species, age, weight, and general condition of the subject, as well as the particular composition being administered and its mode of administration. An appropriate dose can be determined by one of ordinary skill in the art. In some embodiments, the subject is administered about 1 μ g to about 100 μ g of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure. In some embodiments, the subject is administered about 1 μ g to about 50 μ g, about 1 μ g to about 25 μ g, or about 1 μ g to about 5 μ g of the chimeric influenza A virus neuraminidase (NA) protein, virus like particle (VLP), or pharmaceutical composition according to the present disclosure.

[0103] In some embodiments, the dose administered to the subject is about 0.01–10 mg/kg, 0.01–9 mg/kg, 0.01–8 mg/kg, 0.01–7 mg/kg, 0.01–6 mg/kg, 0.01–5 mg/kg, 0.01–4 mg/kg, 0.01–3 mg/kg, 0.01–2 mg/kg, 0.01–1 mg/kg, 0.05–10 mg/kg, 0.05–9 mg/kg, 0.05–8 mg/kg, 0.05–7 mg/kg, 0.05–6 mg/kg, 0.05–5 mg/kg, 0.05–4 mg/kg, 0.05–3 mg/kg, 0.05–2 mg/kg, 0.05–1 mg/kg, 0.1–10 mg/kg, 0.1–9 mg/kg, 0.1–8 mg/kg, 0.1–7 mg/kg, 0.1–6 mg/kg, 0.1–5 mg/kg, 0.1–4 mg/kg, 0.1–3 mg/kg, 0.1–2 mg/kg, 0.1–1 mg/kg, 1–10 mg/kg, 1–9 mg/kg, 1–8 mg/kg, 1–7 mg/kg, 1–6 mg/kg, 1–5 mg/kg, 1–4 mg/kg, 1–3 mg/kg, 1–2 mg/kg, or 0.05–2 mg/kg. In some embodiments, the dose administered to the subject is about 0.05–2 mg/kg mass of the subject.

[0104] The above disclosure generally describes the present disclosure. A more specific description is provided below in the following examples. The examples are described solely for the purpose of illustration and are not intended to limit the scope of the present disclosure. Changes in form and substitution of equivalents are contemplated as circumstances suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitation.

EXAMPLES

Materials and Methods for Examples 1–4

Plasmids

[0105] Codon-optimized FLAG-tagged NA sequences were cloned into pCAGGS expression vector. Sequences were obtained from GenBank with accession numbers: **Ca09-N1**: ACP44181.1; **PR8-N1**: ABD77678.1; **X31-N2**: Q91MA2.1; **X47-N2**: AAB03361.1; **X79-N2**: ADJ41808.1; and **N9**: ARG43209.1.

Cell Culture

[0106] HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) at 37°C and 5% CO₂, with antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin [Pen-Strep]; Gibco) and 10% iron supplemented bovine calf serum (ISBCS; HyClone).

Antibodies

[0107] Primary mouse-FLAG monoclonal antibody was purchased from Millipore. Secondary Alexa Fluor goat anti-mouse monoclonal antibody was purchased from Invitrogen. Primary-conjugated Alexa Fluor monoclonal antibody was purchased from Biolegend.

Transfection

[0108] HEK293T cells were transfected in 6-well plates 20-24 hours post seeding. Media was replaced with 1mL of OptiMEM containing 3 µg of plasmid DNA mixed with 12 µl of 1mg/mL PEI. 4 hours post transfection, 0.5 mL of complete media was added to transfection. Cells and supernatants were collected at 24 hours post transfection.

VLP Purification

[0109] HEK293T cells were transfected with 12 µg of PEI and 3 µg of plasmid DNA. Cell supernatant was collected at 24 hpt from cells and centrifuged at 300 g for 5 minutes at 4°C. Supernatant containing VLPs was centrifuged over a 20% sucrose cushion in NTE buffer at 110,000 g for 1.5 hours at 4°C. The pellets were then resuspended in loading dye with 5% BME and frozen at -20° C until loading in SDS-PAGE.

Cell Lysates

[0110] HEK293T cells from 6-well plates were lysed on ice in 200 µl of RIPA buffer supplemented with protease inhibitor cocktail and vortexed every 10 minutes for 30 minutes. Lysates were centrifuged 15,000 g for 20 minutes at 4°C.

Cell Surface Expression

[0111] Transfected cells were collected and stained with 1:1000 dilution of primary-conjugated Alexa Fluor 647 monoclonal antibody for 1 hour at 4°C. Cells were washed 3 times using FACS buffer (1% FBS in PBS). Cells were analyzed using the Millipore Guavasoft flow cytometer; 10,000 cells were analyzed for every sample.

Western Blot

[0112] To denature samples, 5% BME was added with loading dye and heated for 5 minutes at 95°C. Non-denatured samples received loading dye with no BME and were not heated, were loaded in 10% SDS-PAGE, and run for 1.5 hours at 100V.

Example 1 – N9 Displays a High-Budding Phenotype Compared to N1

[0113] N1, N2, and N9 sequences were codon-optimized and cloned into high-expression plasmid backbones for the most efficient production in mammalian cell culture (FIG. 1A). Two NAs derived from H1N1 viruses (Ca09 and PR8), three NAs derived from H3N2 viruses (X31, X47, and X79), and one NA derived from an H7N9 virus were evaluated. All proteins were assessed for whole-cell expression 24 hours post transfection (hpt) in HEK293T cells. Virus-like particles (VLPs) were collected, purified, and analyzed them via Western blotting (FIG. 1B). In alignment with other studies, NA autonomously forms VLPS independently of other viral proteins (Lai et al., “Formation of virus-like particles from human cell lines exclusively expressing influenza neuraminidase,” *J. Gen. Virol.* 91(Pt 9):2322-2330 (2010), which is hereby incorporated by reference in its entirety). Densitometric analysis was used to establish a budding index (VLP/CL) (FIG. 1B). Although little variation of total NA protein expression in cell lysate was observed, the amount of NA in VLPs had significant differences. N9 was found to increase the budding index >30-fold compared to than selected N1 and N2 serotypes (FIG. 1C).

Example 2 – High-Budding Phenotype of N9 can be Transferred to NA by Swapping TMDs

[0114] Next, Ca09-N1 was further characterized, as it is highly relevant to current human strains and has a very low budding index (FIG. 1B, FIG. 1C). As the sequences of NA domains can vary greatly, how similar Ca09-N1 was to the high-budding N9 was investigated (FIG. 2A). The cytoplasmic tail maintains complete identity between Ca09-N1 and N9. However, the transmembrane domain (TMD) and stalk domain are very different, sharing only ~20% and ~30% identity, respectively. The head was relatively well conserved, maintaining ~50% identity (FIG. 2A). To investigate whether the high-budding phenotype of the N9 could be transferred to a low-budding NA, multiple chimeric NA proteins were created using the TMD and/or stalk domains of the N9 strain (FIG. 2B). To remove variability of differences in codon-optimization, the codon-optimized sequences were kept identical for all versions of the chimeras as they are in their respective parents. Due to ambiguity in the length of the stalk domain, a short stalk (SS) and a long stalk (LS) version were created. Similar total protein expression was observed in the CL (FIG. 2C, FIG. 2D). In contrast, the VLP fraction showed substantial differences. The most significant increase in budding occurred in the N1/N9_{TMD} chimera, with a budding index ~30-fold higher than the Ca09-N1 parent (FIG. 2D). However, in all chimeras in which the N9 stalk was included, significantly less NA was observed in VLPs than that of the N1/N9_{TMD} (FIG. 2D). Interestingly, the N1/N9_{SS-TMD} chimera did contribute to budding, albeit to a lesser degree. The budding index was ~4-fold increased over the Ca09-N1 parent, indicating roles of both the stalk

and the TMD (FIG. 2D). Altogether, the data indicate that the high-budding phenotype of N9 can be transferred to N1 by swapping the TMD.

Example 3 – Stalk Domains, but not TMDs, may Contribute to Trafficking

[0115] It was next investigated whether the budding phenotypes could be due to trafficking to the cell surface. To do this, transfected cells were collected and stained with primary-conjugated antibody. Next, flow cytometry was used to measure relative cell-surface expression (CSE) levels. CSE levels were normalized to the total expression in CL to generate a graph of trafficking (FIG. 3A). Significant differences in CSE and trafficking of NAs was observed. The TMD chimera had significantly increased CSE compared to N1 parent, and similar CSE to N9. The N1/N9_{SS} and N1/N9_{SS-TMD} did not have significant differences in CSE compared to N1 or N9. However, the N1/N9_{LS} and N1/N9_{LS-TMD} had significantly reduced CSE (FIG. 3A). Despite these differences, this does not correlate with the budding phenotype observed, even when VLPs are normalized to CSE (FIG. 3B) indicating that budding phenotypes may be impacted from trafficking to the membrane but are not dependent on CSE.

Example 4 – TMD and Stalk Domain do not Alter the Strength of Oligomerization or Enzymatic Activity

[0116] Next, the stability of the proteins was investigated by looking at oligomerization in a non-denaturing Western blot (FIG. 3A). In CL, N9 had a significantly higher strength of oligomers, showing a higher proportion of dimers than Ca09-N1, and a significantly decreased proportion of monomers (FIG. 3B, FIG. 3C). However, N9 did not have a significant difference in relative strength of tetramers. There was no significant difference between Ca09-N1 and the TMD chimera in any form of oligomerization. There was not a significant difference in the strength of oligomers for the N1/N9_{TMD}, N1/N9_{SS}, or N1/N9_{SS-TMD} chimeras. However, a significant decrease in the percent of monomers for the N1/N9_{LS} and N1/N9_{LS-TMD} chimeras was observed (FIGS. 3B-D). Inclusion of the TMD or stalk in any combination did not lead to replicating an N9-like phenotype, indicating that oligomerization strength may stem largely from interactions within the head domain. Ultimately, the strength of oligomers does not seem to correlate with an increased budding phenotype.

Discussion of Examples 1–4

[0117] Current vaccination strategies provide insufficient protection and are not well-suited to prevent pandemic outbreaks. Existing strategies elicit immune responses against the highly variable HA glycoprotein, resulting in limited protection and a need for annual vaccination. To effectively prevent seasonal epidemics and pandemics, a new vaccine strategy

that provides long-lasting protection against a broad range of IAV serotypes is urgently needed. Current influenza vaccines provide responses primarily to HA (Wohlbold et al., “Vaccination with Adjuvanted Recombinant Neuraminidase induces broad Heterologous, but not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice,” *mBio* 6(2):e02556 (2015), which is hereby incorporated by reference in its entirety). Due to this, yearly vaccinations that provide sub-optimal efficacy are necessary. NA specific vaccination has been shown to provide protection from challenge, even in just in the passive transfer of serum (Wohlbold et al., “Vaccination with Adjuvanted Recombinant Neuraminidase induces broad Heterologous, but not Heterosubtypic, Cross-Protection against Influenza Virus Infection in Mice,” *mBio* 6(2):e02556 (2015), which is hereby incorporated by reference in its entirety). One major limitation to NA vaccines that has plagued vaccination potential is protein production. The use of baculovirus insect cell culture has been adopted to overcome these production hurdles. However, production of vaccine-grade VLPs in insect cell culture are not optimal. Post-translational modification, such as glycosylation, can alter protein folding, epitope availability, and function (Liu et al., “Use of Baculovirus Expression System for Generation of Virus-Like Particles: Successes and Challenges,” *Protein Expr. Purif.* 90(2):104-116 (2013); Gavrilov et al., “Effects of Glycosylation on Antigenicity and Immunogenicity of Classical Swine Fever Virus Envelope Proteins,” *Virology* 420(2):135-145 (2011); and Dowling et al., “Influences of Glycosylation on Antigenicity, Immunogenicity, and Protective Efficacy of Ebola virus GP DNA Vaccines,” *J. Virol.* 81(4):1821-1837 (2007), which are hereby incorporated by reference in their entirety). Production of NA VLPs may be highly strain and serotype dependent. The Examples of the present disclosure demonstrate that that the TMD of N9 can confer a high-budding phenotype to N1 and report a novel method for significantly increasing production NA VLPs in mammalian cell culture.

[0118] Multiple NAs from 3 serotypes were analyzed: N1, N2 and N9. NAs are divided into two groups defined mainly by structure, with N1 belonging to group 1 and N2/N9 belonging to group 2 (Gamblin and Skehel, “Influenza Hemagglutinin and Neuraminidase Membrane Glycoproteins,” *J. Biol. Chem.* 285(37):28403-28409 (2010), which is hereby incorporated by reference in its entirety). Similar levels of expression for all strains and serotypes were observed in the CL, but the levels of NA in the VLP fraction had significant differences. Although there were significant differences in the budding indices between some strains within the N1 and N2 serotypes, N9 stood out as an outlier, being significantly higher than all other NAs. The differences between the Ca09 N1 and the N9 sequences were evaluated, as the Ca09 NA is most relevant to currently circulating human IAVs. The cytoplasmic tail, transmembrane, stalk, and head domains have previously been implicated in translocation, apical sorting, budding, and

oligomerization. However, the cytoplasmic tail is identical between all strains tested, indicating that it does not contribute to this phenotypic difference. The most significant variations between Ca09-N1 and N9 occurred in the TMD, sharing only ~20% identity, followed by the stalk domain with ~30% identity, and finally the head domain, maintaining ~50% identity. The investigation was focused on the transmembrane and stalk domains, as they are the most divergent between Ca09-N1 and N9.

[0119] All chimeric proteins were based on the Ca09-N1 parent, with domains swapped from the N9. Due to ambiguity in the length of the stalk, stalks of two different lengths were utilized: a short stalk (SS) and a long stalk (LS). Both chimeric and parent NAs expressed similarly in the CL, however, had significant differences in VLP expression were observed. A budding index was calculated by dividing band intensity of VLPs by CL. From this, one chimera in particular was significantly increased over the N1 parent. The N1/N9_{TMD} budding index was increased by ~50-fold. Although significant increases with the N1/N9_{SS-TMD} were observed, they were significantly less than just the N1/N9_{TMD}. This may imply that the stalk domain in general does not contribute to the budding phenotype seen and may even be inhibitory.

[0120] As NA buds from the cell surface, whether this phenotype was due to trafficking and expression on the cell surface was investigated. N1/N9_{TMD} was observed to have higher expression on the cell surface than the N1 parent, however, this increase in expression does not reflect the much more significant increase of budding. This implies that the N9 TMD plays major roles in the budding phenotype. Indeed, it has been reported that the NA TMDs are responsible for apical sorting and lipid raft association (Barman and Nayak, "Analysis of the Transmembrane Domain of Influenza Virus Neuraminidase, A Type II Transmembrane Glycoprotein, for Apical Sorting and Raft Association," *J. Virol.* 74(14):6538-6545 (2000), which is hereby incorporated by reference in its entirety). Significantly decreased CSE was observed for N1/N9_{LS} and N1/N9_{LS-TMD} chimeras. As chimeras containing the SS did not show a difference in CSE, the extra stalk length of the chimeras containing the LS may contribute to trafficking.

[0121] As the TMD and stalk domains have been implicated to play roles in oligomerization strength, how this was impacted by the chimeric proteins was investigated (da Silva et al., "Assembly of Subtype 1 Influenza Neuraminidase is Driven by Both the Transmembrane and Head Domains," *J. Biol. Chem.* 288(1):644-653 (2013), which is hereby incorporated by reference in its entirety). Interestingly, a significant difference in the relative strength of oligomers for the N1/N9_{TMD}, N1/N9_{SS}, or N1/N9_{SS-TMD} chimeras was not observed. However, a significant decrease in the percent of monomers for the N1/N9_{LS} and N1/N9_{LS-TMD}

chimeras was observed, indicating that strength of oligomerization may contribute to trafficking differences. N9 high percent of dimers compared to N1, chimeric NAs did not reflect a difference in dimerization to their N1 parent, indicating that the head domain also plays major roles in oligomerization.

5 **[0122]** Overall, both the TMD and stalk were observed to have roles in budding and trafficking phenotypes. Importantly, the high budding phenotype of N9 can be transferred to N1 by swapping TMDs. This may aide in the production of mammalian-expressed NA VLPs, while preserving the immunodominant ectodomain.

10 **[0123]** Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions, and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

WHAT IS CLAIMED:

1. A chimeric influenza A virus (IAV) neuraminidase (NA) protein comprising:
a cytoplasmic tail;
5 a transmembrane domain;
a stalk domain; and
a head domain, wherein said cytoplasmic tail, said stalk domain, and said head domain are from a first influenza A virus neuraminidase protein,
wherein said transmembrane domain is from a second influenza A virus
10 neuraminidase protein which is different from the first influenza A virus, said second influenza A virus neuraminidase protein having a budding phenotype which is greater than the budding phenotype of the first influenza A virus neuraminidase protein.
2. The chimeric influenza A virus neuraminidase protein of claim 1, wherein the
15 first influenza A virus neuraminidase protein is a low-budding neuraminidase protein.
3. The chimeric influenza A virus neuraminidase protein of claim 1 or claim 2, wherein the second neuraminidase protein is from a medium-budding neuraminidase protein or high-budding neuraminidase protein.
20
4. The chimeric influenza A virus neuraminidase protein of any one of claims 1 to 3, wherein said first influenza A virus neuraminidase protein is an N1 neuraminidase protein.
5. The chimeric influenza A virus neuraminidase protein of claim 4, wherein the
25 N1 neuraminidase protein is Ca09-N1.
6. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein the first influenza A virus neuraminidase protein is Ca09-N1.
- 30 7. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein
said cytoplasmic tail has an amino acid sequence of SEQ ID NO: 25;
said stalk domain has an amino acid sequence of SEQ ID NO: 26; and
said head domain has an amino acid sequence of SEQ ID NO: 27.

8. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein said second influenza A virus neuraminidase protein is an N9 neuraminidase protein.

5 9. The chimeric influenza A virus neuraminidase protein of claim 8, wherein the N9 neuraminidase protein is a high budding NA.

10 10. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein said second neuraminidase protein is A/Yunnan/0129/2017.

11. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein the transmembrane domain has an amino acid sequence of SEQ ID NO: 28.

15 12. The chimeric influenza A virus neuraminidase protein of any of the preceding claims wherein said cytoplasmic tail, said stalk domain, and said head domain are from an influenza A virus N1 neuraminidase protein and wherein said transmembrane domain is from an influenza A virus N9 neuraminidase protein.

20 13. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein the chimeric neuraminidase protein has an amino acid sequence of SEQ ID NO: 29.

25 14. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein the chimeric influenza A virus neuraminidase protein has a budding phenotype which is greater than the budding phenotype of the first influenza A virus neuraminidase protein.

30 15. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein the chimeric influenza A virus neuraminidase protein has a budding phenotype which is at least about 10-fold greater, at least about 20-fold greater, at least about 30-fold greater, at least about 40-fold greater, or at least about 50-fold greater than the budding phenotype of the first influenza A virus neuraminidase protein.

16. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein said stalk domain of the chimeric influenza A virus neuraminidase is a short

stalk domain, optionally wherein the stalk domain has the amino acid sequence of SEQ ID NO: 30.

17. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein said stalk domain of the chimeric influenza A virus neuraminidase comprises a long stalk domain, optionally wherein the stalk domain has the amino acid sequence of SEQ ID NO: 31.

18. The chimeric influenza A virus neuraminidase protein of any of the preceding claims, wherein the head domain is at the C-terminus of the neuraminidase protein, the head domain is connected at its amino end to the stalk domain of the neuraminidase protein, the stalk domain is connected at its amino end to the transmembrane domain, the transmembrane domain is connected at its amino end to the cytoplasmic domain, and/or the cytoplasmic domain is at the amino-terminus of the neuraminidase protein.

19. A nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein of any one of claims 1 to 18.

20. A vector encoding a chimeric influenza A virus neuraminidase protein, wherein the vector comprises the nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein of claim 19.

21. The vector of claim 20, wherein the vector further comprises:
a promoter and
a termination sequence, wherein said promoter and said termination sequence are operably coupled to the nucleic acid molecule encoding the chimeric influenza A virus neuraminidase protein.

22. A host cell comprising the nucleic acid molecule of claim 16 or the vector of claim 20 or claim 21.

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

24. The host cell of claim 23, wherein the host cell is mammalian.

25. The host cell of claim 24, wherein the mammalian cell is selected from the group consisting of Human embryonic kidney (HEK) cells (e.g., HEK293 cells, HEK293F cells, HEK293T cells), Chinese hamster ovary (CHO) cells (e.g., CHO Toronto/CHO pro 3, CHO K1, DG 44, DXB11, CHO K1 SV, CHOK1SV GS-KO), HeLa cells, COS cells, PC3 cells, African
5 Green Monkey kidney cells (e.g., Vero cells), baby hamster kidney (BHK) cells (e.g., BHK21), PER.C6® cells, and Madin-Darby canine kidney (MDCK) cells (e.g., MDCK cells, MDCK-HA cells, MDCK/London cells, MDCK.2 cells, MDCK SIAT-1 cells).

26. A virus-like particle produced by the host cell of any one of claims 22 to 25.

27. A virus-like particle comprising the chimeric influenza A virus neuraminidase protein of any one of claims 1 to 18.

28. A method of producing a virus-like particle comprising a chimeric influenza
15 A virus neuraminidase protein, said method comprising:
providing the vector of claim 18 or claim 19;
transfecting a host cell with the vector; and
culturing the host cell in a cell culture comprising a supernatant under conditions effective to express the chimeric influenza A virus neuraminidase protein and allow budding of
20 virus-like particles comprising tetramers of the chimeric influenza A virus neuraminidase proteins into the supernatant.

29. The method of claim 28, wherein the host cell is a mammalian host cell.

30. The method of claim 29, wherein the host cell is selected from the group consisting of Human embryonic kidney (HEK) cells (e.g., HEK293 cells, HEK293F cells, HEK293T cells), Chinese hamster ovary (CHO) cells (e.g., CHO Toronto/CHO pro 3, CHO K1, DG 44, DXB11, CHO K1 SV, CHOK1SV GS-KO), HeLa cells, COS cells, PC3 cells, African
25 Green Monkey kidney cells (e.g., Vero cells), baby hamster kidney (BHK) cells (e.g., BHK21), PER.C6® cells, and Madin-Darby canine kidney (MDCK) cells (e.g., MDCK cells, MDCK-HA cells, MDCK/London cells, MDCK.2 cells, MDCK SIAT-1 cells).

31. A method of producing a virus-like particle comprising a chimeric influenza A virus neuraminidase protein, said method comprising:
35 providing the host cell of any one of claims 18 to 23;

culturing the host cell in a cell culture comprising a supernatant under conditions effective to express the chimeric influenza A virus neuraminidase protein and to allow budding of virus-like particles comprising tetramers of the chimeric influenza A virus neuraminidase proteins into the supernatant.

5

32. The method of any of claims 28 to 31 further comprising:
recovering the virus-like particles from the supernatant.

10

33. The method of claim 32, wherein said recovering comprises:
collecting the supernatant containing the virus-like particles and
centrifuging the collected supernatant to pellet the virus-like particles.

15

34. A pharmaceutical composition comprising:
a plurality of virus-like particles of claims 26 to 27 and
a pharmaceutically acceptable carrier.

35. The pharmaceutical composition of claim 34, wherein the composition is
lyophilized.

20

36. A method of eliciting an immune response in a subject, said method
comprising:
administering an effective amount of the chimeric influenza A virus
neuraminidase (NA) protein of claims 1 to 18, the virus like particle of claim 26 or claim 27, or
the pharmaceutical composition of claims 34 or 35 to a subject, wherein said administering is
effective to elicit an immune response in the subject.

25

37. The method of claim 36, wherein the said immune response is a humoral
immune response.

30

38. The method of claim 36 or claim 37, wherein said immune response is
effective to reduce the risk of infection or prevent infection with an influenza virus.

39. The method of any of claims 36 to 38, wherein the subject is a mammal.

35

40. The method of claim 39, wherein the subject is a human.

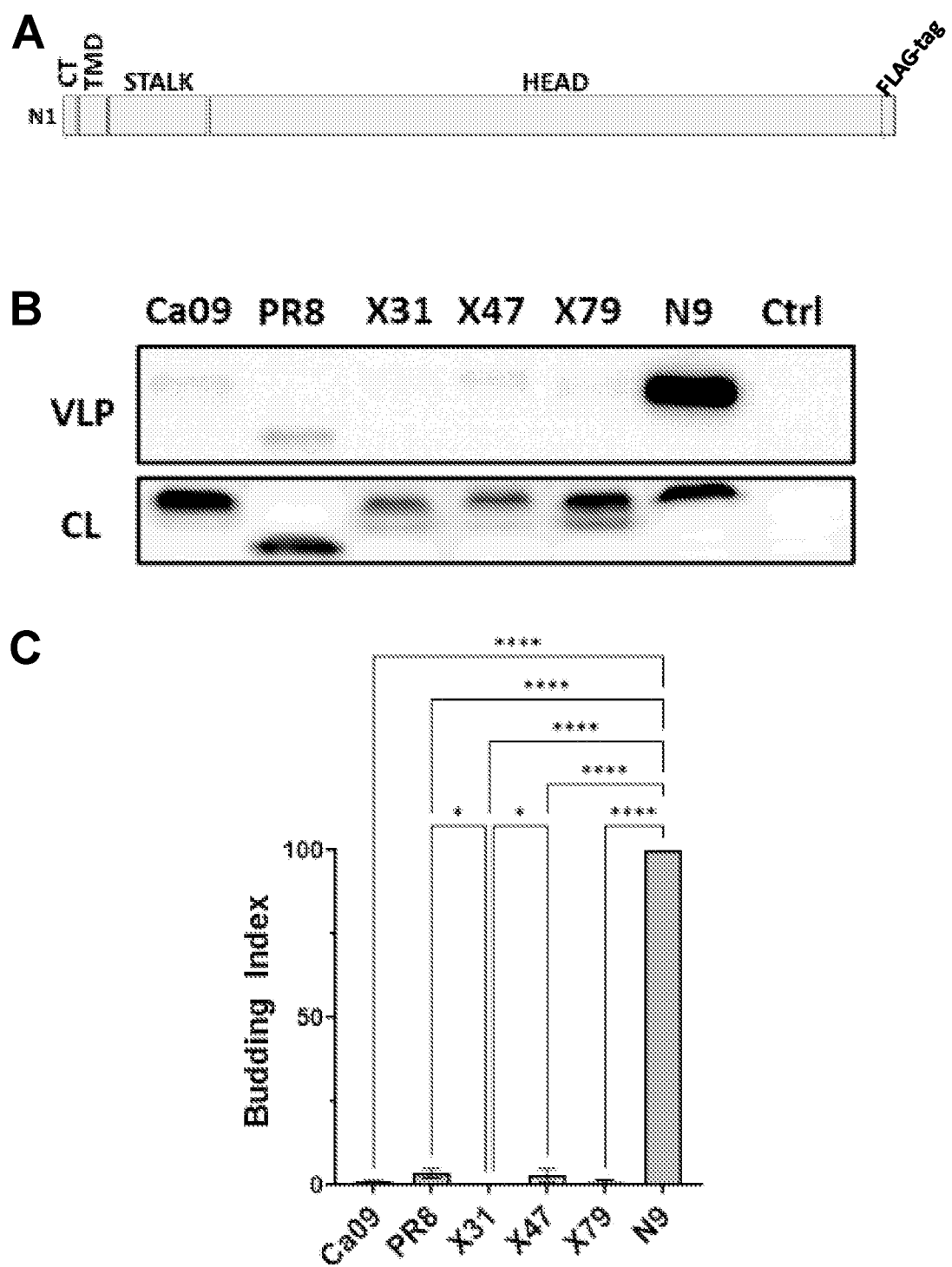
41. The method of claim 40, wherein the subject is an infant, an adolescent, or an adult.

5 42. The method of any of claims 36 to 41, wherein the chimeric influenza A virus neuraminidase (NA) protein, virus like particle, or the pharmaceutical composition is administered at a dose of 1 to 50 micrograms.

10 43. The method of any of claims 36 to 42, wherein the chimeric influenza A virus neuraminidase (NA) protein or the pharmaceutical composition is administered subcutaneously, parenterally, intravenously, intramuscularly, orally, intraperitoneally, transdermally, topically, by oral or nasal inhalation, or by direct injection to one or more cells, tissues, or organs.

15 44. The method of any one of claims 36 to 43, wherein the chimeric influenza A virus neuraminidase (NA) protein or the pharmaceutical composition is administered as a single dose.

20 45. The method of any one of claims 36 to 43, wherein the chimeric influenza A virus neuraminidase (NA) protein or the pharmaceutical composition is administered in multiple doses.



FIGS. 1A-1C

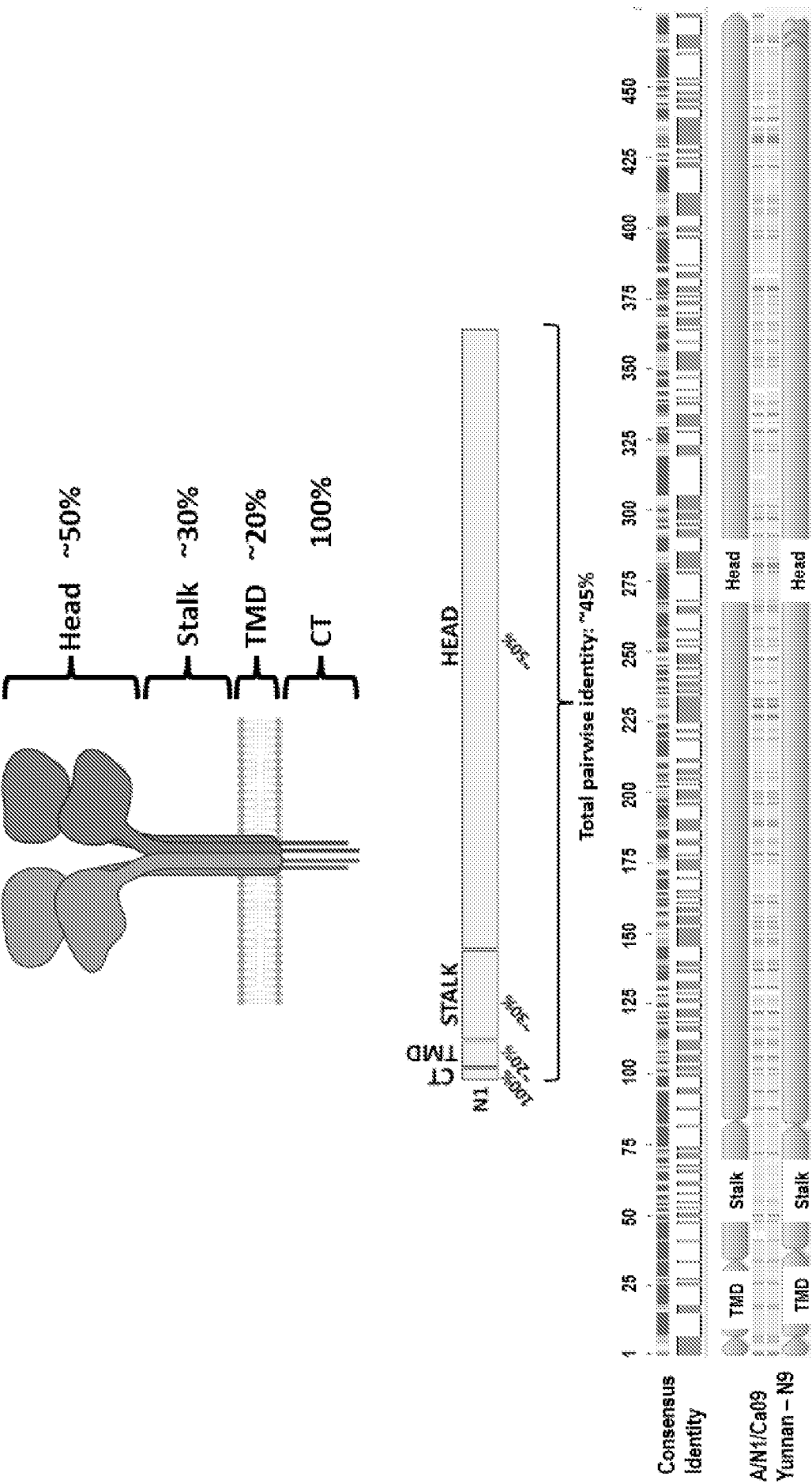
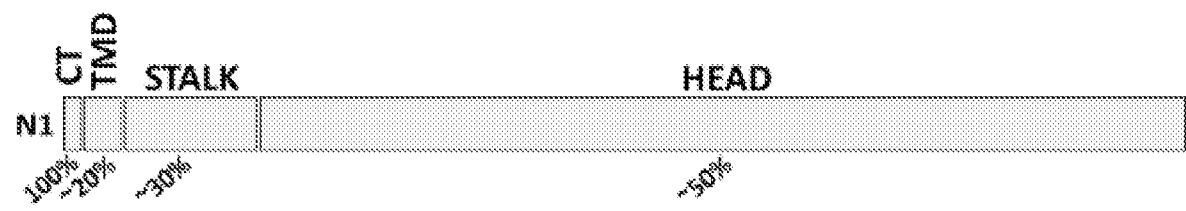
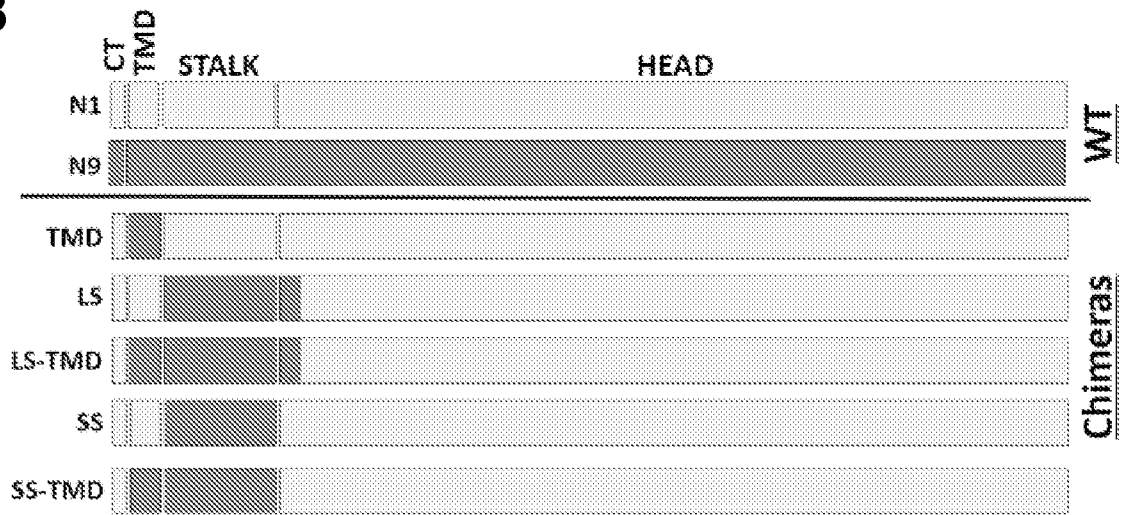


FIG. 1D

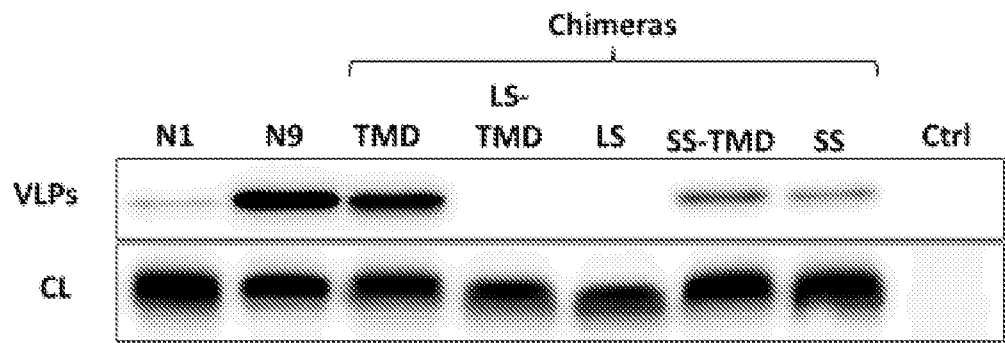
A



B



C



FIGS. 2A-2C

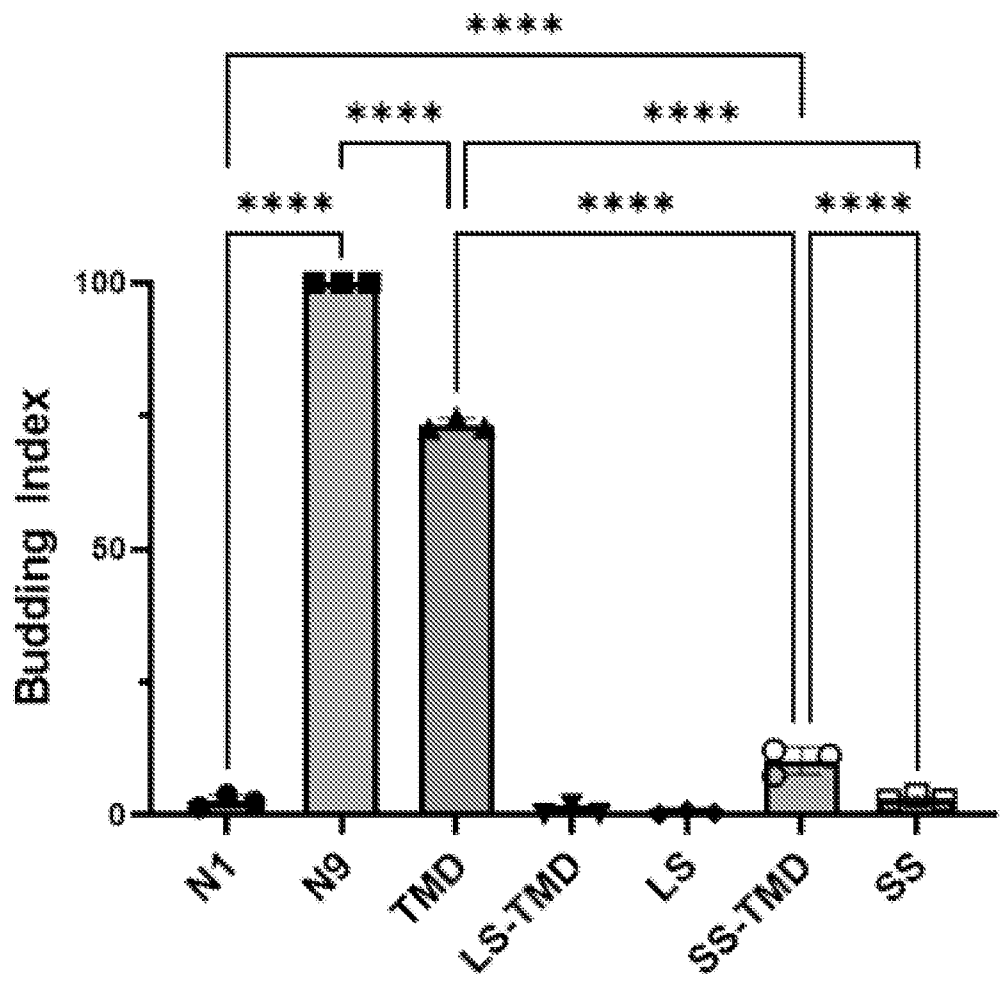
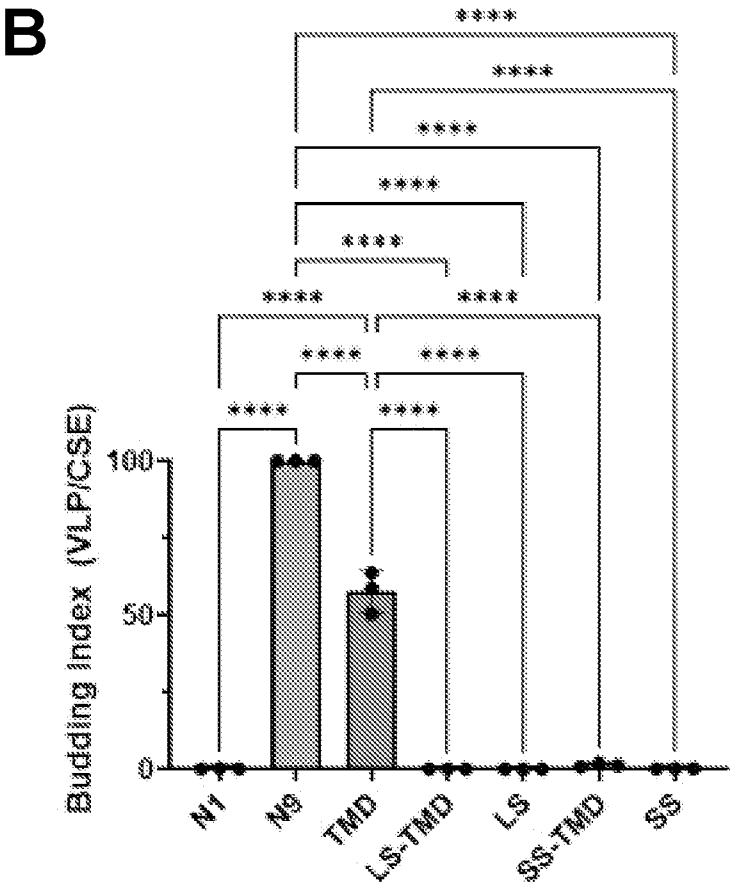
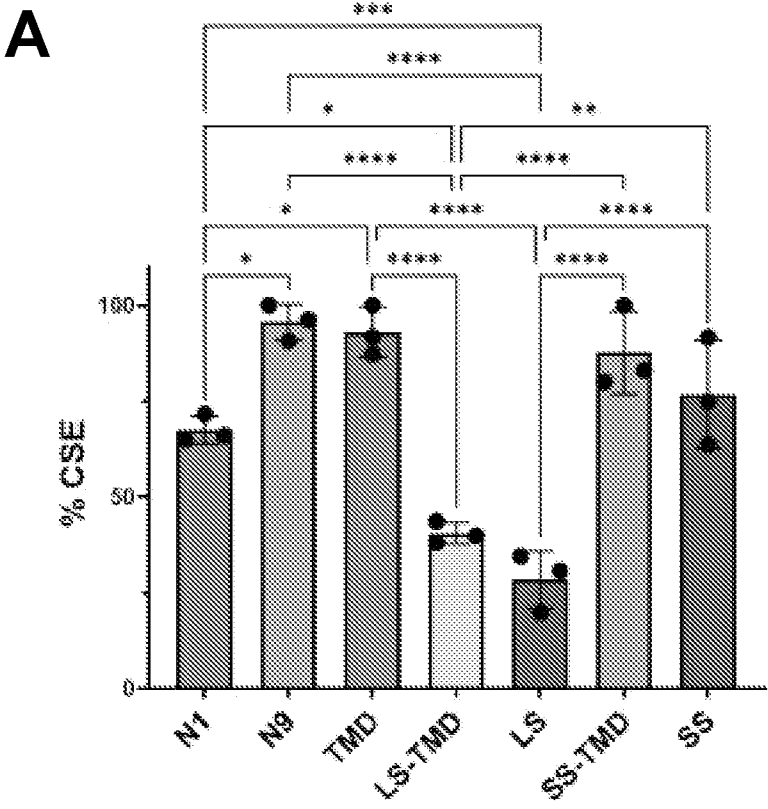


FIG. 2D



FIGS. 3A-3B

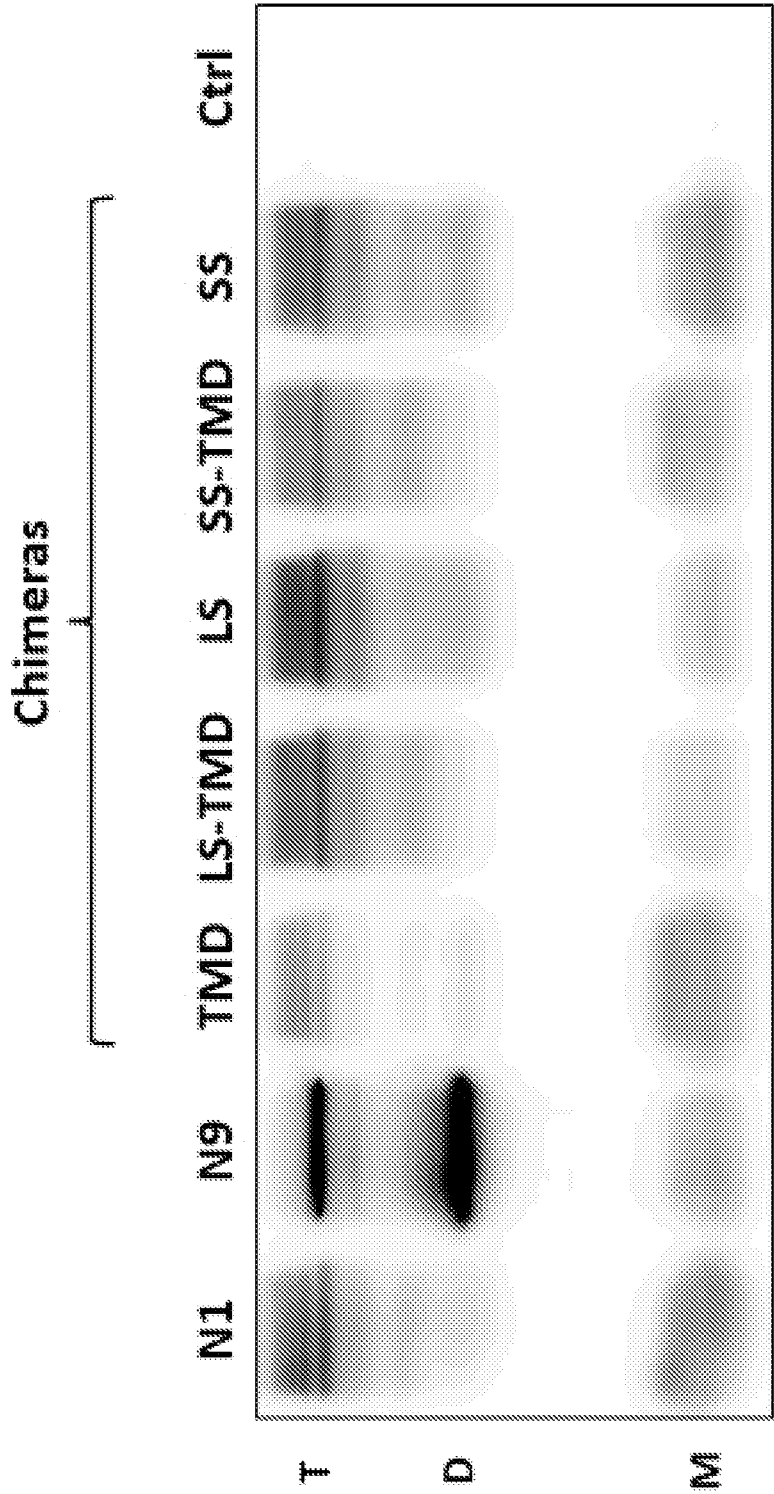
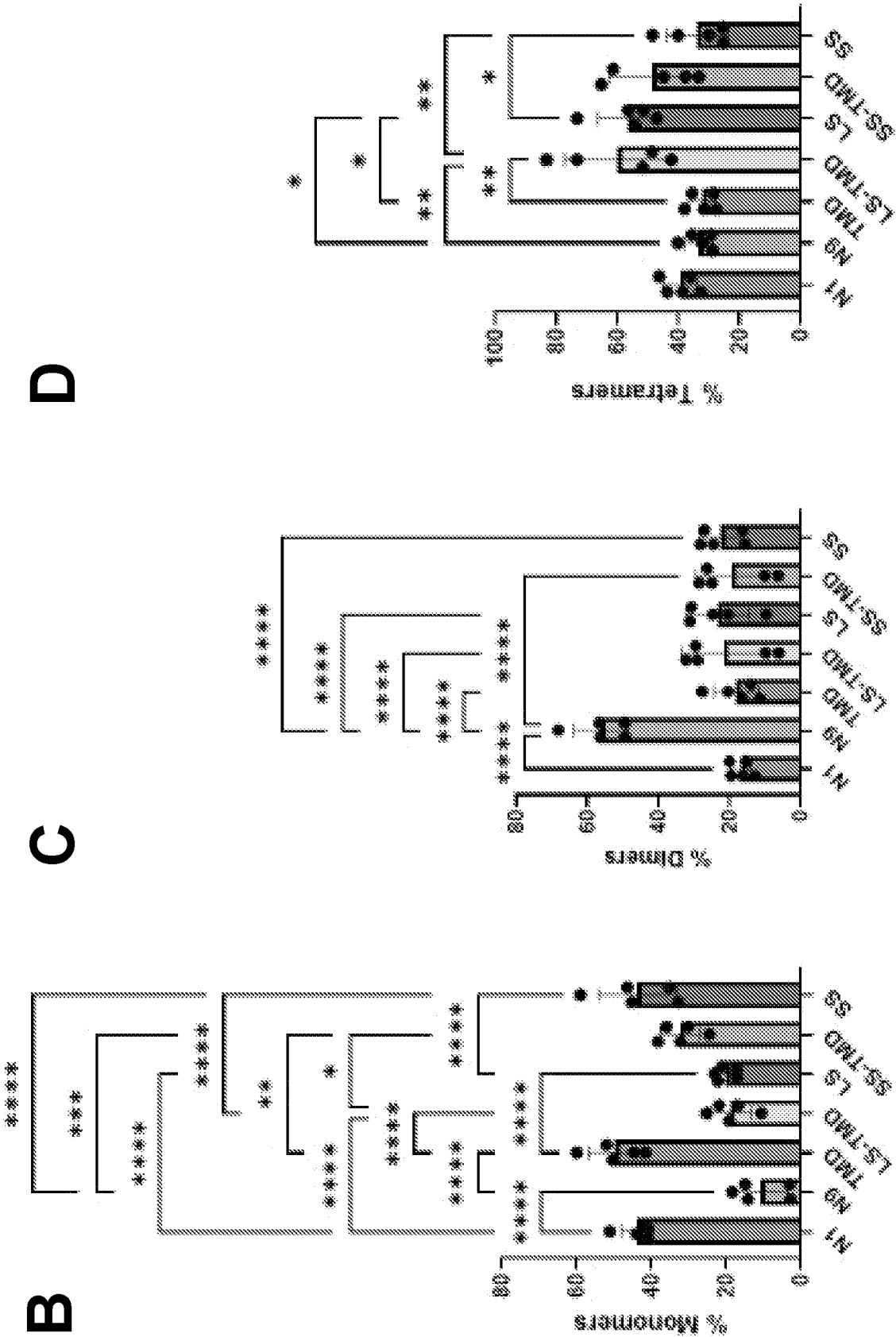


FIG. 4A



FIGS. 4B-4C

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US23/84866

A. CLASSIFICATION OF SUBJECT MATTER

IPC - INV. A61K 39/145; C07K 14/11; C12N 7/04; C12N 9/24 (2023.01)

ADD.

CPC - INV. A61K 39/145; C07K 14/11; C12N 7/04; C12N 9/2402

ADD. A61K 2039/5258; C07K 2319/03; C12N 2760/16134; C12Y 302/01018

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2022/0249652 A1 (ICAHN SCHOOL OF MEDICINE AT MOUNT SINAI) 11 August 2022; paragraph [0010]	1-3
A	WO 2021/081120 A1 (ICAHN SCHOOL OF MEDICINE AT MOUNT SINAI) 29 April 2021; paragraph [0012]	1-3
A	PLANT ET AL. "Chimeric neuraminidase and mutant PB1 gene constellation improves growth and yield of H5N1 vaccine candidate virus" pages 752-755. Journal of General Virology. Vol. 96. 2015; page 752, column 1, paragraph 1; DOI: 10.1099/jgv.0.000025	1-3
A	DA SILVA ET AL. "The Influenza Virus Neuraminidase Protein Transmembrane and Head Domains Have Coevolved" pages 1094-1104. Journal of Virology. Vol. 89, No. 2. January 2015; page 1095, column 2, paragraph 2; Fig. 1A; DOI: 10.1128/JVI.02005-14	1-3
☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 March 2024 (27.03.2024)

Date of mailing of the international search report

APR 10 2024

Name and mailing address of the ISA/

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents

P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Shane Thomas

Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US23/84866

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
- a. ☐ forming part of the international application as filed.
- b. ☒ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☒ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US23/84866

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-45
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.