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(54) Title: CHIMERIC HEMAGGLUTININ PROTEIN AND A VACCINE COMPOSITION COMPRISING THE SAME

(57) Abstract: Provided is a chimeric hemagglutinin (HA) protein including an HA1 subunit and an HA2 subunit, in which the HA1 subunit is composed of a first domain derived from a parental HA1 subunit of a first subtype influenza virus and a second domain derived from a parental HA1 subunit of a second subtype influenza virus. The chimeric HA protein has improved thermal stability and can be used in a vaccine composition for preventing influenza virus infection. Also provided is a method of inducing an immune response against an influenza virus in a subject in need thereof that includes administering the chimeric HA protein to the subject, thereby conferring protection against the influenza virus infection on the subject.

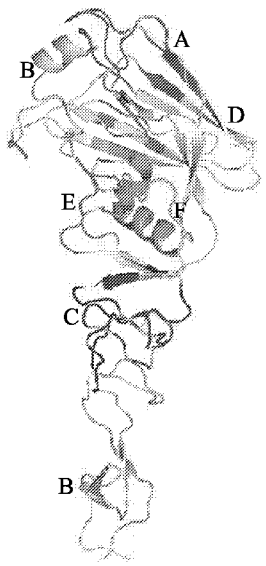


FIG. 1B

WO 2021/055679 A1

[Continued on next page]

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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# CHIMERIC HEMAGGLUTININ PROTEIN AND A VACCINE COMPOSITION COMPRISING THE SAME

## BACKGROUND

### 1. Technical Field

The present disclosure relates to a chimeric hemagglutinin (HA) protein exhibiting high stability and immunogenicity that can be used to produce effective vaccines. The present disclosure further relates to a method for preventing viral infection, e.g., influenza virus infection.

### 2. Description of Related Art

Influenza virus infection has long been a serious epidemic disease among humans. Seasonal influenza viruses result in approximately 3 to 5 million severe infection cases and 290,000 to 650,000 deaths worldwide annually[1], while occasional emergence of human-infected avian influenza viruses (e.g., H5N1 and H7N9) further threatens human health and economics.

During influenza virus infection, the glycoprotein hemagglutinin (HA) is a key antigen determinant that is responsible for binding to host cell surface receptors (e.g., sialic acid-containing glycans) and subsequent endosomal membrane fusion. The HA protein of an influenza virus is comprised of HA1 and HA2 subunits, of which the HA1 subunit contains a receptor binding site (RBS) for binding to sialic acid receptors, whereas the HA2 subunit contains a fusion peptide and transmembrane domain (TM) that are responsible for trimerization[2]. Accordingly, the HA protein has become a primary target for developing anti-influenza drugs and vaccines.

However, researchers developing influenza vaccines readily encounter problems owing to the instability of the HA protein[3-6]. For instance, HA stability affects vaccine utility, as it significantly reflects vaccine immunogenicity and storage life[4, 7]. Unstable HAs may easily be subject to a post-fusion conformation or even dissociate into monomers that induces antibodies that recognize invalid epitopes, instead of the functionally neutralizing antibodies required to tackle infection, thereby resulting in not only reduced protection but also shortened vaccine shelf-life[3-6].

In 2013, the devastating H7N9 influenza virus was identified in China, which induced high mortality[9]. This virus has continued to circulate in China and has resulted in epidemics across the country. The H7N9 virus has been classified as a highly pathogenic avian influenza virus (HPAIV), so that effective vaccines for the H7N9 influenza virus are urgently needed for human and veterinary use[10]. However, the HA protein of the H7N9 influenza virus is relatively unstable that potentially reduces the efficacy of the respective vaccine for effective immunization. Therefore, there exists an unmet need for an effective vaccine that exhibits improved stability of the HA protein from an influenza virus without adversely impairing its immunogenicity.

## SUMMARY

The present disclosure provides a chimeric HA protein that is a stabilizing chimeric antigen while maintaining proper immunogenicity, and thus is useful for producing an effective vaccine against an influenza virus. In the present disclosure, the HA1 subunit in the chimeric HA protein is a chimeric subunit, which means that the protein domains thereof are derived from different HA1 subunits, such as H7 and H3 subtypes.

In one embodiment of the present disclosure, the chimeric HA protein comprises an HA1

subunit and an HA2 subunit, wherein the HA1 subunit is composed of a first domain derived from a parental HA1 subunit of a first subtype influenza virus and a second domain derived from a parental HA1 subunit of a second subtype influenza virus. In another embodiment, the second domain in the HA1 subunit is at least one portion of an HA structural region selected from the group consisting of a fusion peptide pocket, an HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit, an HA1-HA1 interface, and an HA1-HA2 interface. In one embodiment, the HA2 subunit is an HA2 subunit of the first subtype influenza virus.

In one embodiment of the present disclosure, the first subtype influenza virus and the second subtype influenza virus are independently selected from the group consisting of H1 to H18 subtype influenza viruses, provided that the first subtype influenza virus and the second subtype influenza virus are different. In another embodiment, the first subtype influenza virus and the second subtype influenza virus are independently selected from the group consisting of H1, H2, H5, H6, H8, H9, H11 to H13, and H16 to H18 subtype influenza viruses, provided that the first subtype influenza virus and the second subtype influenza virus are different. In yet another embodiment, the first subtype influenza virus and the second subtype influenza virus are independently selected from the group consisting of H3, H4, H7, H10, H14, and H15 subtype influenza viruses, provided that the first subtype influenza virus and the second subtype influenza virus are different.

In one embodiment of the present disclosure, the first subtype influenza virus is an H7 subtype influenza virus, and the second subtype influenza virus is an H3 subtype influenza virus.

In one embodiment of the present disclosure, the parental HA1 subunit of the first subtype influenza virus is derived from an H7N9 influenza virus. In another embodiment, the parental HA1 subunit of the first subtype influenza virus has an amino acid sequence of SEQ ID NO: 1.

In one embodiment of the present disclosure, the parental HA1 subunit of the second

subtype influenza virus is derived from an H3N2 influenza virus. In another embodiment, the parental HA1 subunit of the second subtype influenza virus has an amino acid sequence of SEQ ID NO: 2.

In one embodiment of the present disclosure, the HA1 subunit of the chimeric HA protein has an amino acid identity less than 100% as compared with the parental HA1 subunit of the first subtype influenza virus. In another embodiment, the HA1 subunit has an amino acid identity of at least 30% as compared with the parental HA1 subunit of the first subtype influenza virus. In yet another embodiment, the amino acid identity of the HA1 subunit to the parental HA1 subunit of the first subtype influenza virus is between 70% and 95%. In still another embodiment, the amino acid identity of the HA1 subunit to the parental HA1 subunit of the first subtype influenza virus is between 88% and 91%.

In one embodiment of the present disclosure, the chimeric HA protein comprises at least one of: (1) the fusion peptide pocket of the chimeric HA protein that includes Ala, Thr, Leu, Asn, Lys, and Arg; (2) the HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit of the chimeric HA protein that includes Asp and Ser; (3) the HA1-HA1 interface of the chimeric HA protein that includes Asn and Ser; and (4) the HA1-HA2 interface of the chimeric HA protein that includes Arg, Val, Lys, Ile, Tyr, and Ala.

In one embodiment of the present disclosure, the second domain in the HA1 subunit is derived from at least one amino acid, at least one peptide or a combination thereof selected from the group consisting of positions #11-#13, #21, #25, #27, #29, #31-#34, #37, #42, #44-#45, #46-#50, #53-#56, #58, #185-#189, #193, #216-#217, #219, #228, #268-#269, #271-#274, #276, #278-#280, #282-#285, #287, #289-#292, #297-#302, #304, #307, #312-#313, #315, #321, and #326-#329 of SEQ ID NO: 2; for example, positions #11-#13 refer to a peptide with three consecutive amino acids at positions 11 to 13 of SEQ ID NO: 2, and #21 refers to a single

amino acid at position 21 of SEQ ID NO: 2.

In one embodiment of the present disclosure, the chimeric HA protein comprises at least one of: (1) the fusion peptide pocket including Ala, Thr, Leu, Asn, Thr, Lys, and Arg at positions 1, 2, 3, 303, 304, 306, and 312 in SEQ ID NO: 12, respectively; (2) the HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit, the HA1 region including Asp and Ser at positions 22 and 35 in SEQ ID NO: 12, respectively; and (3) the HA1-HA1 interface including Asn and Ser at positions 207 and 210 in SEQ ID NO: 12, respectively.

In one embodiment of the present disclosure, the chimeric HA protein comprises the HA1-HA2 interface including Arg, Val, Lys, Ile, Tyr, Ala, and Lys at positions 259, 287, 289, 290, 292, 294, and 297 in SEQ ID NO: 13, respectively.

In one embodiment of the present disclosure, the HA1 subunit of the chimeric HA protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 8.

In one embodiment of the present disclosure, a vaccine composition is provided. The vaccine composition comprises the chimeric HA protein of the present disclosure and a pharmaceutically acceptable carrier and/or an adjuvant. In another embodiment, the adjuvant is at least one of a squalene adjuvant, a cytokine adjuvant, a lipid adjuvant and a Toll-like receptor (TLR) ligand.

In one embodiment of the present disclosure, the chimeric HA protein in the vaccine composition is present in an effective amount to prevent influenza virus infection, or to induce an immune response against an influenza virus in a subject in need thereof.

In one embodiment of the present disclosure, the vaccine composition is suitable for administration via intranasal, intramuscular, intravenous, intra-arterial, intraperitoneal, intrathecal, intraventricular, subcutaneous and mucosal routes.

In one embodiment of the present disclosure, a method is provided for inducing an immune

response against an influenza virus in a subject in need thereof. In one embodiment, the method is provided for conferring protection against influenza virus infection on the subject. In one embodiment of the present disclosure, the influenza virus is an H1N1, H1N2, H2N2, H3N2, H5N1, H5N2, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7 or H10N8 influenza virus. In another embodiment, the influenza virus is an H7N9 influenza virus.

In one embodiment of the present disclosure, the method comprises administering the vaccine composition of the present disclosure to the subject. In another embodiment, the subject is a vertebrate. In still another embodiment, the subject is a mammal, such as a human.

In the present disclosure, the chimeric HA proteins provided by the present disclosure not only achieve the construction of a more stable HA antigen, but also facilitate effective vaccine improvements to fight against infection of influenza viruses.

## BRIEF DESCRIPTION OF THE DRAWINGS

The present disclosure can be more fully understood by reading the following descriptions of the embodiments, with reference made to the accompanying drawings.

FIGs. 1A and 1B illustrate the non-contiguous SCHEMA recombination of H7-HA1 and H3-HA1. FIG. 1A shows the non-identical amino acids between the H7-HA1 and H3-HA1 subunits divided by SCHEMA into six blocks (presented by different colors) according to their known protein structures and sequence alignment. FIG. 1B depicts the six blocks shown in the H7-HA1 structure. The division of these six blocks are consistent across individual domains of the 3D structure, except for the block B that is divided into two sub-domains which are non-continuous across the peptide sequence. H7-HA1 represents the HA1 subunit from the H7 protein (i.e., the HA protein from an H7 subtype influenza virus), and H3-HA1 represents the HA1 subunit from the H3 protein (i.e., the HA protein from an H3 subtype influenza virus).

FIG. 2 illustrates different constructs of the HA1 subunits and their 3D structures, wherein H7-HA1 refers to the HA1 subunit from the H7 subtype influenza virus; H3-HA1 refers to the HA1 subunit from the H3 subtype influenza virus; H7-HA2 refers to the HA2 subunit from the H7 subtype influenza virus; H3-HA2 refers to the HA2 subunit from the H3 subtype influenza virus; and rA-HA1 to rF-HA1 refer to the six chimeric HA1 subunits.

FIGs. 3A and 3B show the recombinant baculovirus constructions for the expression of parental and chimeric HA proteins in full length. FIG. 3A shows the constructs of expression vectors of the full-length parental and chimeric HAs. All constructs are driven by the polyhedrin promoter (p-polh), fused with an N-terminal GP64 signal peptide (SP) and a hexameric histidine tag (6H), and include a pag promoter (*p-pag*) driving the DsRed gene as a reporter. The six chimeric HA proteins, FrA to FrF, were constructed by fusing the HA2 subunit from the H7 subtype influenza virus to the C-termini of individual rA to rF chimeric HA1 subunits. WT-DR virus was generated by an empty vector containing only the DsRed reporter as a negative control. FIG. 3B shows the Western blot analysis of the full-length HA constructs. Insect cells were infected by recombinant baculoviruses expressing each of the HA constructs at a multiplicity of infection (MOI) equal to 1. Cell lysates were harvested at 2 days post infection (d.p.i.) before performing Western blot by using the anti-His antibody. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected by the anti-GAPDH antibody as a loading control.

FIGs. 4A to 4D illustrate the 3D structures of different HA structural regions of the chimeric HA proteins. FIG. 4A shows the fusion peptide pocket of the chimeric HA protein FrB, wherein Ala 1, Thr 2, Leu 3, Asn 303, Thr 304, Lys 306, and Arg 312 refer to the amino acids and their positions of the chimeric rB-HA1 subunit (SEQ ID NO: 12). FIG. 4B shows the HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit of the chimeric HA

protein FrB, wherein Asp 22 and Ser 35 refer to the amino acids and their positions of the chimeric rB-HA1 subunit (SEQ ID NO: 12). FIG. 4C shows the HA1-HA1 interface of the chimeric HA protein FrB, wherein Asn 207 and Ser 210 refer to the amino acids and their positions of the chimeric rB-HA1 subunit (SEQ ID NO: 12). FIG. 4D shows the HA1-HA2 interface of the chimeric HA protein FrC, wherein Arg 259, Val 287, Lys 289, Ile 290, Tyr 292, Ala 294, and Lys 297 refer to the amino acids and their positions of the chimeric rC-HA1 subunit (SEQ ID NO: 13). The HA1-HA2 interface shown in FIG.4D is present by two diagrams, in order to clearly illustrate the multiple amino acids.

FIG. 5 shows the determination of cell-surface expression of HA constructs by immunofluorescence assay, wherein Sf21 cells infected by recombinant viruses with different HA constructs at MOI equal to 1 were fixed by 4% paraformaldehyde at 2 d.p.i., before HA proteins were stained by the primary anti-His antibody and secondary Alexa Fluor 488 antibody (green fluorescence). 4',6-diamidino-2-phenylindole (DAPI) staining (blue fluorescence) was used as a counterstain. Red fluorescence was from the DsRed reporter gene carried by the individual recombinant viruses.

FIG. 6 shows the determination of the localization of FrA, FrD, FrE, and FrF by immunofluorescence assay, wherein Sf21 cells infected by the recombinant viruses expressing FrA, FrD, FrE, or FrF at MOI equal to 1 were fixed at 2 d.p.i., and half of the samples were permeabilized by 0.2% Triton. Localization of HA proteins was detected by the primary anti-His antibody and secondary Alexa Fluor 488 antibody (green fluorescence), with DAPI (blue fluorescence) as a counterstain. Proper red fluorescence expression from the DsRed reporter gene indicated successful virus infection.

FIG. 7 shows the characterization of the chimeric HAs by H7 antibody recognition, wherein ELISA analysis revealed the Sf21 cells infected by baculoviruses expressing one of the

HA constructs as antigens. Data are expressed as mean values ± standard deviation (SD), representing three replicates from three independent experiments. * refers to the significant difference ($p < 0.05$) versus the value of FH7; ns: not significant.

FIGs. 8A and 8B show the characterization of the chimeric HAs by the hemagglutination assay. FIG. 8A illustrates the schematic hemagglutination assay, wherein in the absence of HA-expressing samples, red blood cells precipitate in the V-bottom wells that forms a red-colored dot at the center of each well; upon encountering HA-expressing samples, the red blood cells clump with HA-displaying insect cells to form lattices and produce a diffuse pale red signal in V-bottom wells. FIG. 8B shows the hemagglutination assay of the recombinant baculovirus-infected Sf21 cells, wherein the HA titer of each sample was determined as the reciprocal of the highest dilution with remaining HA activity. Phosphate-buffered saline (PBS) refers to the buffer-only control; HA: purified H7 protein (representing 500 ng in the first row); Non: non-infected Sf21 cells.

FIG. 9 shows the thermal hemagglutination assay to determine the stability of HAs, wherein Hi5 cells infected with different recombinant baculoviruses were prepared with an initial HA titer of 64, and incubated at 50°C for the indicated time periods (0, 5, 10, 20, 30, 60, 90, and 120 minutes). After cooling down to 4°C, HA titers of cell samples were measured by the hemagglutination assay. Data are expressed as mean values ± SD, representing three replicates from three independent experiments. * refers to significant difference ($p < 0.05$) versus the titer of FH7 at each time point.

FIGs. 10A and 10B show that the antibodies elicited by FrB and FrC recognize an original FH7 antigen and inhibit H7N9 virus infection. FIG. 10A shows sera (1:10,000) from mice (n = 5) immunized intraperitoneally with purified FH7, FrB, or FrC proteins or PBS and then collected at week 6 and week 8 after primary immunization, before measuring the specific anti-HA IgG

antibody binding levels by indirect ELISA against purified FH7 protein. Data are expressed as mean values ± SD for five mice in each group with technical triplicates. FIG. 10B shows the microneutralization assay of FH7-, FrB- or FrC-immunized mouse sera against an H7N9 influenza virus (the A/Taiwan/01/2013 strain) infection. The mouse sera were serially diluted 2-fold (initial concentration 1:10), and mixed with 10 times the 50% tissue culture infective doses ($TCID_{50}$) of the H7N9 influenza virus to determine microneutralization titers (the reciprocal of the highest dilution without CPE) in the infected MDCK cells. Data are expressed as mean values ± SD for five mice in each group with technical quadruplicates. * refers to significant difference ($p < 0.05$) versus PBS; † refers to significant difference ($p < 0.05$) versus FH7 at designated time points.

## DETAILED DESCRIPTION OF THE EMBODIMENTS

The following examples are used for illustrating the present disclosure. A person skilled in the art can easily conceive the other advantages and effects of the present disclosure, based on the disclosure of the specification. The present disclosure can also be implemented or applied as described in different examples. It is possible to modify or alter the following examples for carrying out this disclosure without contravening its spirit and scope, for different aspects and applications.

It is further noted that, as used in this disclosure, the singular forms “a,” “an,” and “the” include plural referents unless expressly and unequivocally limited to one referent. The term “or” is used interchangeably with the term “and/or” unless the context clearly indicates otherwise.

The present disclosure is directed to chimeric HA proteins and their uses as stable HA antigens in a vaccine composition for prevention of viral infections.

The chimeric HA protein of the present disclosure comprises a chimeric HA1 subunit, which comprises a first domain derived from a parental HA1 subunit of a first subtype influenza virus and a second domain derived from a parental HA1 subunit of a second subtype influenza virus.

In one embodiment of the present disclosure, the first subtype influenza virus and the second subtype influenza virus are independently selected from the group consisting of H1 to H18 subtype influenza viruses, provided that the first subtype influenza virus and the second subtype influenza virus are different. In another embodiment of the present disclosure, the first subtype influenza virus and the second subtype influenza virus are independently selected from Group I influenza viruses, such as H1, H2, H5, H6, H8, H9, H11 to H13, and H16 to H18 subtype influenza viruses, or Group II influenza viruses, such as H3, H4, H7, H10, H14, and H15 subtype influenza viruses.

The term "chimeric HA protein," "chimeric protein," or "chimeric subunit" as used herein refers to a single polypeptide unit that comprises at least two heterological domains joined by a peptide bond(s), wherein the different domains are not naturally occurring within the same polypeptide unit. As to the amino acid sequence of the chimeric protein, each heterological domain may correspond to non-continuous amino acids or a number of peptide fragments. These non-continuous amino acids and peptide fragments may assemble as an integrated and structurally interacting domain. For instance, such chimeric proteins may be obtained by expression of a cDNA construct or by protein synthesis methods known in the art.

For example, the chimeric HA1 subunits of the present disclosure may contain two domains derived from the HA protein subtypes H7 and H3 (i.e., the HA proteins from the H7 subtype influenza virus and the H3 subtype influenza virus, respectively), which means that such chimeric subunits may contain a plurality of non-continuous amino acids and/or a plurality

of peptide fragments homological to a naturally occurring HA1 subunit of the HA protein from the H7 subtype influenza virus, and a plurality of non-continuous amino acids and/or a plurality of peptide fragments homological to a naturally occurring HA1 subunit of the HA protein subtype H3.

The term "domain" or "protein block" as used herein refers to a set of at least one amino acid, at least one peptide, or a combination thereof in a protein. That is to say, a domain of a protein may include only one amino acid, a plurality of non-continuous amino acids, only one peptide, a plurality of peptide, or a combination thereof. For example, the first domain in the chimeric HA1 subunit of the present disclosure may be composed of amino acid(s) which is/are derived from the parental HA1 subunit of the first subtype influenza virus. In addition, some of the amino acid(s) in the domain of the protein may constitute a portion of a structural region of the protein.

In one embodiment of the present disclosure, the HA1 subunit of the chimeric HA protein is derived from the parental HA1 subunits, e.g., the naturally occurring HA1 subunits of the H7N9 influenza virus and the H3N2 influenza virus. In another embodiment, the H7N9 influenza virus is an A/Anhui/1/2013 strain, and the H3N2 influenza virus is an A/Hong Kong/1/1968 strain.

In one embodiment of the present disclosure, the parental HA1 subunit of the first subtype influenza virus includes an amino acid sequence at least 70%, 75%, 80%, 85%, 88%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 1. In another embodiment, the parental HA1 subunit of the first subtype influenza virus has the amino acid sequence of SEQ ID NO: 1.

In one embodiment of the present disclosure, the parental HA1 subunit of the second subtype influenza virus includes an amino acid sequence at least 70%, 75%, 80%, 85%, 88%,

90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence of SEQ ID NO: 2. In another embodiment, the parental HA1 subunit of the second subtype influenza virus has the amino acid sequence of SEQ ID NO: 2.

In one embodiment of the present disclosure, the amino acid identity of the HA1 subunit of the chimeric HA protein as compared with the parental HA1 subunit of the first subtype influenza virus is from 30% to less than 100%, and the chimeric HA protein containing such HA1 subunit has higher thermal stability and comparable immunogenicity in comparison with the HA protein containing the parental HA1 subunit. In one embodiment, the HA1 subunit of the chimeric HA protein has less than 95% amino acid identity as compared with the parental HA1 subunit of the first subtype influenza virus. In another embodiment, the HA1 subunit of the chimeric HA protein has at least 70% amino acid identity as compared with the parental HA1 subunit of the first subtype influenza virus. In yet another embodiment, the amino acid identity of the HA1 subunit of the chimeric HA protein as compared with the parental HA1 subunit of the first subtype influenza virus is between 71% and 94%, such as 75%, 80%, 85%, 88%, 89%, 90%, 91%, 92%, 93% and 94%.

In one embodiment of the present disclosure, the HA1 subunit of the chimeric HA protein includes an amino acid sequence at least 70%, 75%, 80%, 85%, 88%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 8, and has the same functions as SEQ ID NOs: 3 to 8, respectively. In another embodiment, the HA1 subunit of the chimeric HA protein has an amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 8.

In one embodiment of the present disclosure, the chimeric HA protein includes an amino acid sequence at least 70%, 75%, 80%, 85%, 88%, 90%, 92%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence selected from the group consisting of SEQ ID NOs: 11 to

16, and has the same functions as SEQ ID NOs: 11 to 16, respectively. In another embodiment, the chimeric HA protein has an amino acid sequence selected from the group consisting of SEQ ID NOs: 11 to 16.

In one embodiment of the present disclosure, the second domain in the chimeric HA1 subunit is at least one portion of an HA structural region selected from the group consisting of a fusion peptide pocket, an HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit, an HA1-HA1 interface, and an HA1-HA2 interface.

For example, the HA structural regions may include: (1) a fusion peptide pocket, i.e., a region near the "F domain" of the HA1 subunit which surrounds the fusion peptide; (2) an HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit; (3) an HA1-HA2 interface, i.e., a region of the interface between the HA1 receptor-binding domain protomers; or (4) an HA1-HA1 interface, i.e., a region between the receptor-binding domain, esterase subdomain, helix C, and loop B.

In one embodiment of the present disclosure, the chimeric HA protein may comprise at least one of: (1) the fusion peptide pocket of the chimeric HA protein that includes Ala, Thr, Leu, Asn, Lys, and Arg; (2) the HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit of the chimeric HA protein that includes Asp and Ser; (3) the HA1-HA1 interface of the chimeric HA protein that includes Asn and Ser; and (4) the HA1-HA2 interface of the chimeric HA protein that includes Arg, Val, Lys, Ile, Tyr, and Ala.

In one embodiment of the present disclosure, a portion of the amino acid residue(s) in the chimeric HA1 subunit is replaced by the amino acid residue(s) at corresponding position(s) of the parental HA1 subunit of the second subtype influenza virus, so as to form the second domain in the chimeric HA1 subunit. In another embodiment, the second domain is derived from at least one amino acid, at least one peptide or a combination thereof selected from the group

consisting of positions #11-#13, #21, #25, #27, #29, #31-#34, #37, #42, #44-#45, #46-#50, #53-#56, #58, #185-#189, #193, #216-#217, #219, #228, #268-#269, #271-#274, #276, #278-#280, #282-#285, #287, #289-#292, #297-#302, #304, #307, #312-#313, #315, #321, and #326-#329 of SEQ ID NO: 2.

The term "sequence identity," "amino acid identity," or "homology" as used herein refers to describe sequence relationships between two or more nucleotide sequences or amino acid sequences. The percentage of the "sequence identity" between two sequences is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window may comprise additions or deletions (e.g., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. A sequence that is identical at every position in comparison to a reference sequence is said to be identical to the reference sequence and *vice-versa*. Included are nucleotides or polypeptides having at least about 70%, 75%, 80%, 85%, 88%, 90%, 92%, 95%, 97%, 98%, 99% or 100% sequence identity to any of the reference sequences described herein (see, e.g., Sequence Listing), where the polypeptide variant maintains at least one biological activity or function of the reference polypeptide.

In certain embodiments of the present disclosure, vaccine compositions are provided for primary immunization of a subject against influenza. In the present disclosure, the vaccine composition may include a chimeric HA protein of the present disclosure as a main antigen for use in the reduction of severity or for use in the prevention of influenza infections. In other

embodiments, methods for reducing the severity or preventing influenza infections by using the vaccine composition of the present disclosure are also provided.

In one embodiment of the present disclosure, the chimeric HA protein in the vaccine composition is present in an effective amount to prevent influenza virus infection, or to induce an immune response against an influenza virus in a subject in need thereof. In another embodiment, the vaccine composition is administered in an amount sufficient to elicit an immune response against an influenza virus, such as the H7N9 subtype, in a subject in need thereof.

In one embodiment of the present disclosure, the vaccine composition may further comprise a pharmaceutically acceptable carrier and/or an adjuvant. In another embodiment, the adjuvant is at least one of a squalene adjuvant, a cytokine adjuvant, a lipid adjuvant and a Toll-like receptor (TLR) ligand. The examples of the TLR ligand includes, but are not limited to, 3-deacylated monophoshoryl lipid A (3D-MPL), lipopolysaccharide (LPS), muramyl dipeptide (MDP), and CpG motifs. In yet another embodiment, the vaccine composition administered to the subject comprises a mixture of the chimeric HA protein as an antigen and the adjuvant at a weight ratio of 10:1 to 1:10.

The term "pharmaceutically acceptable carrier" as used herein refers to any and all solvents, dispersion media, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like which may be appropriate for administration of the vaccine composition of the present disclosure. The pharmaceutically acceptable carrier useful for the present disclosure may include, but not be limited to, a preservative, a suspending agent, a tackifier, an isotonicity agent, a buffering agent, a humectant, and a combination thereof.

In one embodiment of the present disclosure, the vaccine composition may be administered by any suitable delivery route, such as intranasal, intramuscular, intravenous,

intra-arterial, intraperitoneal, intra-thecal, intraventricular, subcutaneous and mucosal routes. In another embodiment, the vaccine composition of the present disclosure is administered to a subject under conditions sufficient to prevent influenza infection in the subject.

In one embodiment of the present disclosure, a method is provided for inducing an immune response against an influenza virus in a subject in need thereof. In another embodiment, the influenza virus is H1N1, H1N2, H2N2, H3N2, H5N1, H5N2, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7 or H10N8 subtype influenza virus. In yet another embodiment, the subject is a vertebrate. In still another embodiment, the subject is a mammal, such as a human.

In one embodiment of the present disclosure, the method comprises administering a vaccine composition comprising a chimeric HA protein to a subject in need thereof, wherein the chimeric HA protein comprises an HA1 subunit composed of a first domain and a second domain, and wherein the first domain is derived from a parental HA1 subunit of a first subtype influenza virus, and the second domain is derived from a parental HA1 subunit of a second subtype influenza virus. In another embodiment, the parental HA1 subunit of the first subtype influenza virus is derived from an H7N9 subtype influenza virus, such as an A/Anhui/1/2013 strain, and may have the amino acid sequence of SEQ ID NO: 1. In yet another embodiment, the parental HA1 subunit from the HA protein of the second subtype influenza virus is derived from an H3N2 influenza virus, such as an A/Hong Kong/1/1968 strain, and may have the amino acid sequence of SEQ ID NO: 2.

In one embodiment of the present disclosure, the amino acid identity of the HA1 subunit of the chimeric HA protein as compared with the parental HA1 subunit from the HA protein of the first subtype influenza virus is between 70% and 95%. In another embodiment, the HA1 subunit of the chimeric HA protein comprises an amino acid sequence selected from the group

consisting of SEQ ID NOs: 3 to 8.

In one embodiment of the present disclosure, the chimeric HA protein further comprises an HA2 subunit that may be an HA2 subunit from the first subtype influenza virus, such as an H7N9 subtype influenza virus.

In an embodiment of the present disclosure, the chimeric HA protein has improved stability and enhanced immunogenicity in comparison with the naturally occurring HA protein of the influenza virus, such as the H7N9 subtype influenza virus, such that the chimeric HA protein of the present disclosure may be used as a better vaccine antigen.

Many examples have been used to illustrate the present disclosure. The examples below should not be taken as a limit to the scope of the present disclosure.

## EXAMPLES

### Materials and Methods

The materials and methods used in the following Examples 1-5 were described in detail below. The materials used in the present disclosure but unannotated herein are commercially available.

#### (1) Non-contiguous SCHEMA recombination

The amino acid sequences of H7-HA1 (i.e., the HA1 subunit from the H7 protein) and H3-HA1 (i.e., the HA1 subunit from the H3 protein) were aligned by ROMALS3D[17]. The resulting alignment and protein structures of H7-HA1 and H3-HA1 were used as input for non-contiguous SCHEMA recombination to create SCHEMA contact maps, in which the SCHEMA algorithm considered any two amino acids as being in contact if any atoms (excluding hydrogen) from the two amino acids were within 4.5 Å of each other. The structure of H7-HA1 was derived

from Protein Data Bank (PDB) Accession No. 4LN6[18] chain A. For H3-HA1, it was PDB Accession No. 4WE4[19] chain A. SCHEMA distributed the non-identical residues of these two HA1s into blocks and calculated the number of disrupted contacts upon block swapping for each chimera (represented as the *E* value) relative to the closest parental protein.

(2) Viral DNA and plasmid DNA

The cDNA sequences of the full-length A/Anhui/1/2013 (H7N9) and A/Hong Kong/1/1968 (H3N2) HA, as well as of the six chimeric HA1s, were synthesized by GenScript, U.S.A. The FH7 and FH3 coding regions including the ectodomain, transmembrane domain, and cytoplasmic tail domain were amplified from the A/Anhui/1/2013 (H7N9) and A/Hong Kong/1/1968 (H3N2) HA cDNAs, respectively, and then inserted along with the AcMNPV GP64 signal peptide and a hexametric histidine tag at the N-terminal into a baculovirus transfer vector, pBacPAK8 (Clontech). The DsRed gene driven by the pag promoter[11, 12] was also inserted into the vector to serve as the reporter gene. Sequences of chimeric HA1 proteins were individually cloned into the transfer vector of FH7 to replace the HA1 portion. The empty vector pBacPAK8 with only the pag-dsRed reporter gene was used as the transfer vector for the WT-DR virus.

(3) Cells and viruses

*Spodoptera frugiperda* IPLB-Sf21 (Sf21) cells were cultured at 26°C in TC100 insect medium (Gibco, Thermo Fisher Scientific) with 10% fetal bovine serum (FBS). Recombinant AcMNPVs were generated by co-transfecting the transfer vector plasmids carrying HA constructs with FlashBAC (Mirus, a modified AcMNPV baculovirus genome) into Sf21 cells by Cellfectin (Life Technologies). The resulting recombinant baculoviruses were propagated in

Sf21 and isolated through end-point dilutions as previously described[20, 21]. *Trichoplusia ni* BTI-TN-5B1-4 (Hi5) cells were cultured at 26°C in ESF serum-free insect cell culture medium (Expression Systems) without adding FBS. Madin-Daby canine kidney (MDCK) cells were cultured in a monolayer at 37°C and 5% $CO_2$ using Dulbecco's Modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% FBS.

(4) Recombinant HA protein expression and Western blotting analysis

Sf21 cells were infected by recombinant viruses at MOI equal to 1 and incubated for 2 days to express the recombinant proteins. The cells were collected, washed with Dulbecco's phosphate-buffered saline (DPBS) to remove the culture medium, and lysed by RIPA Lysis and Extraction Buffer (Thermo Scientific). Equal amounts of cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel (Omic Bio) and Western blotted using mouse anti-His antibody (1:5,000, GeneTex GTX628914) to determine protein expression. Expression of GAPDH for each sample was determined using rabbit anti-GAPDH (10,000, GeneTex GTX100118) as a loading control.

(5) Immunofluorescence assay to detect the expression of HA

Sf21 cells ($1\times10^4$) were seeded into 8-well Millicell EZ slides (Millipore), and the cells were infected with recombinant baculovirus using MOI equal to 1, before fixing cells with 4% paraformaldehyde at 2 d.p.i. For cells requiring additional permeabilization, 0.2% Triton (prepared in DPBS) was added into the cells, and then the cells were incubated for 5 min. After blocking with 3% bovine serum albumin (BSA) in DPBS for 1 h, the cells were incubated with mouse anti-His-tagged antibody (1:5000, GeneTex GTX628914) overnight at 4°C. The cells were washed three times with DPBST (DPBS, plus 0.1% Tween 20) and incubated with 1:200-

diluted Alexa Fluor goat anti-mouse IgG secondary antibody (Invitrogen). Images were obtained with a Zeiss laser confocal microscope (LSM780) and analyzed by ZEN 2010 software (Zeiss).

(6) Cell-based enzyme-linked immunosorbent assay (ELISA)

Sf21 cells were cultured in a 96-well plate and infected with recombinant baculoviruses using MOI equal to 1 to display HA protein antigens on cell surfaces. Culture medium was removed at 3 d.p.i., and the cells were washed by DPBS. The cells were then fixed by 4% paraformaldehyde and permeabilized by 0.2% Triton treatment. The permeabilized cells were incubated with the blocking buffer (3% BSA in DPBS) for 1 h at room temperature. The H7N9 H7-specific neutralizing monoclonal antibody (11082-R002, Sino Biological Inc.) was diluted 1:5,000 in the blocking buffer, added to the cell samples, and then incubated overnight at 4°C. After three washes with 0.1% Tween 20 in PBS (PBST), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (diluted 1:10,000; Merck Millipore) was added to each well for 1 h at room temperate. The samples were washed three times with PBST and the 3,3',5,5'-tetramethyl benzidine (TMB) substrate was then added. Coloring reactions were stopped using 2 M sulfuric acid, and ELISA absorbance was measured at 450 nm. The average read of cell served only as a blank for other samples.

(7) Hemagglutination assay

To ensure higher recombinant protein expression, Hi5 cells were used in the hemagglutination assay. Optimal hemagglutination activity of cell surface-expressed HAs was determined at 5 d.p.i. of recombinant viruses at MOI equal to 0.5. The infected Hi5 cells were collected from the monolayer cultures, and centrifuged to remove the culture medium. The

pelleted cells were suspended in PBS (pH 7.2) plus 0.01% BSA and disrupted by a brief sonication. Fifty microliters of the disrupted cell suspension was added into the V-bottom 96-well plates and serially diluted 2-fold to a final 256-fold dilution. Fifty microliters of 1% turkey erythrocytes (suspended in PBS containing 0.01% BSA) were added into each well and incubated for 1 h at room temperature. The hemagglutination titer was defined as the reciprocal of the highest dilution to agglutinate turkey erythrocytes.

(8) Thermal stability assay measured by loss of hemagglutination titer

The infected Hi5 cell samples exhibiting HA expression were prepared to HA titers of 64 per 50 μL and incubated at 50°C for 0, 5, 10, 20, 30, 60, 90, and 120 min. After being cooled down to 4°C, the samples were subjected to the hemagglutination assay to determine the loss of hemagglutination titer.

(9) Protein purification for mice immunizations

To purify the HA proteins for mice immunization, Hi5 cells were infected by vFH7, vFrB, and vFrC, respectively, at MOI equal to 5. The cells were harvested at 4 d.p.i. by low-speed centrifugation. Cell pellets were treated with I-PER Insect Cell Protein Extraction Reagent (Thermo Scientific) (with the addition of 1% Triton) on ice for 10 min to extract the recombinant HAs. Cell lysates were clarified by centrifugation at 10,000×g for 30 min, and the supernatants were loaded on metal affinity chromatography columns packed with Ni Sepharose 6 Fast Flow resin (GE Healthcare). The columns were washed with carbonate wash buffer (50 mM $NaHCO_3$, 300 mM NaCl, 20 mM imidazole, pH 8), and recombinant HAs were eluted with an elution buffer (50 mM $NaHCO_3$, 300 mM NaCl, 300 mM imidazole, pH 8). The purified proteins were dialyzed in the PBS buffer and then concentrated by Amicon Ultra Centrifugal

Filter Units (Merck Millipore). Protein concentrations were determined by using a Coomassie Plus (Bradford) Assay Kit (Thermo Scientific).

(10) Mice immunizations

All mice for immunization assays were purchased from the Taiwan National Laboratory Animal Center, and the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Academia Sinica, Taiwan. Five female BALB/c mice (6- to 8-weeks-old) per group were immunized intraperitoneally with 30 μg of each purified full-length recombinant protein homogenized with Freund's complete adjuvant. The negative control group was immunized with PBS only. Two boost shots, each of 30 μg antigen in Freund's incomplete adjuvant, were administered 2 and 4 weeks after the primary immunization. Serum was collected from all mice at 6 and 8 weeks after the primary immunization.

(11) Indirect ELISA assay to measure serum H7-specific IgG

Levels of serum IgG-specific antibodies against FH7 antigen were determined for each serum sample by indirect ELISA according to a previously described method[22]. Purified FH7 (20 ng/well) was coated on the 96-well plate overnight at 4°C. After blocking by 3% BSA (in DPBS) for 1 h, mouse sera (1:10,000 dilution) were added to the wells in triplicate and incubated for 2 h at room temperature. The wells were then washed three times with DPBST, before adding goat anti-mouse IgG conjugated with HRP (Merck Millipore) and incubating for 1 h. After three washes by PBST, the TMB substrate was added to each well. The coloring reactions were stopped using 2 M sulfuric acid, and ELISA absorbance was measured at 450 nm using an ELISA plate reader.

(12) Serum microneutralization assay

The A/Taiwan/01/2013 (H7N9) influenza virus was first amplified, and its $TCID_{50}$ was determined in MDCK cells. Collected mouse sera were filtered using a 0.22 μm filter, serially diluted 2-fold (from 1:10 to 1:1,280), mixed with 10 $TCID_{50}$ of H7N9 virus, and incubated at 4°C for 1 h. The mixtures were then transferred to monolayer MDCK cells in 96-well plates and cultured at 37°C. Neutralizing activity was determined at 3 d.p.i. by observing the virus-induced cytopathic effect (CPE), and the microneutralizing titer was defined as the reciprocal of the highest dilution that totally prevented the CPE. For statistical analysis, each serum sample was assessed in quadruplicate.

(13) Statistical analyses

For cell-based ELISA, thermal hemagglutination assays, indirect ELISA, and serum microneutralization assay, each condition was analyzed with at least three replicates (or quadruplicate for the microneutralization assay). All quantitative data are shown as means ± SD (error bars). Statistical analysis was performed using unpaired t-test (Excel 2016 software; Microsoft) for two group comparisons, and $P$-values $< 0.05$ were considered significant.

Example 1: Construction of the chimeric HA1 subunit

For improving stability of the H7 protein (i.e., the HA protein from an A/Anhui/1/2013 strain (the H7N9 subtype)), the H3 protein from an A/Hong Kong/1/1968 strain (the H3N2 subtype) was selected for recombination with the H7 protein, because both the two subtypes belong to group II influenza viruses and the H3 protein (i.e., the HA protein from the H3 subtype influenza virus) is phylogenetically related to the H7 protein (i.e., the HA protein from the H7 subtype influenza virus).

SCHEMA, which is a computational algorithm used in protein engineering to identify fragments of proteins (called as protein blocks or domains) that can be recombined without disturbing the integrity of the three-dimensional structure of the protein in interest, was employed in this Example for construction of the chimeric protein.

The selected H7 and H3 proteins exhibit 49% identity to each other. For instance, the HA1 subunits present 38% identity, whereas the HA2 subunits have 68% identity. Since the HA1 subunit of the HA protein is primarily responsible for sequence divergence and harbors most of the antigenic sites, the HA1 subunit of the H7 protein (H7-HA1; SEQ ID NO: 1) and the HA1 subunit of the H3 protein (H3-HA1; SEQ ID NO: 2) were collected for providing a total of non-identical amino acids for block assignment. The SCHEMA algorithm distributed these non-identical residues into different blocks according to structural adjacency and calculated *E* values representing the number of residue-residue contacts (two amino acids with at least one non-hydrogen atom within 4.5 Å) that would be broken in a chimera upon block swapping between two proteins.

It was decided to divide the HA1 subunits of the H7 and H3 proteins into six blocks (block A to block F), which nearly distribute the 201 non-identical amino acids of the two HA1 subunits evenly. These divisions are non-continuous along the peptide sequence (FIG. 1A), but the amino acids in each block are assembled as an integrated and structurally interacting domain (FIG. 1B). The only exception is block B (the green color shown in FIG. 1B), which SCHEMA further divided it into two sub-blocks; one representing the N and C termini joined together as a sub-block (the lower portion shown in FIG. 1B), and the other comprising the remaining amino acids in the HA head domain (the upper portion shown in FIG. 1B).

Each of the chimeric proteins was designed to solely have one block swapped from the H3 protein and the rest of the protein originated from the H7 protein, which resulted in six

individual clones (designated as rA to rF, Table 1 and FIG. 2). The amino acid sequences of the chimeric HA1 subunits rA to rF are represented by SEQ ID NOs: 3 to 8, respectively.

Table 1. Parental and SCHEMA-derived chimeric HA1 subunits

| Protein | | Inherited block | | | | | | *E* | *m* |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | | |
| Parental | H7 | 7 | 7 | 7 | 7 | 7 | 7 | 0 | 0 |
| | H3 | 3 | 3 | 3 | 3 | 3 | 3 | 0 | 0 |
| Selected chimeras | rA | 3 | 7 | 7 | 7 | 7 | 7 | 15 | 34 |
| | rB | 7 | 3 | 7 | 7 | 7 | 7 | 10 | 32 |
| | rC | 7 | 7 | 3 | 7 | 7 | 7 | 15 | 34 |
| | rD | 7 | 7 | 7 | 3 | 7 | 7 | 27 | 32 |
| | rE | 7 | 7 | 7 | 7 | 3 | 7 | 28 | 32 |
| | rF | 7 | 7 | 7 | 7 | 7 | 3 | 47 | 32 |

Inherited block: the numbers "7" and "3" represent the block origin. For example, rA comprises block A from the H3 protein and the remaining blocks all from the H7 protein.

*E*: the number of residue-residue contacts calculated by SCHEMA that would be broken upon block swapping relative to the closest parental protein.

*m*: the number of amino acid changes relative to the closest parental protein.

Example 2: Generation of the full-length HA expression system

Since the bioactivity of the HA protein primarily relies on its trimeric conformation, the full-length chimeric HA constructs were generated by fusing the chimeric HA1 subunits with an HA2 subunit. The HA2 subunit from the H7 protein was employed and fused to the C-termini

of the six chimeric HA1 subunits to form the full-length constructs (designated as FrA to FrF, respectively). The full-length parental constructs, FH7 and FH3, were constructed using their original HA1 and HA2 sequences, respectively (FIG. 3A). The full-length amino acid sequences of HA1 and HA2 sequences in the parental constructs FH7 and FH3 and the chimeric HA constructs FrA to FrF are represented by SEQ ID NOs: 9 to 16, respectively.

Further, the recombinant baculoviruses, vFH7, vFH3, and vFrA to vFrF, were generated for carrying the respective expression constructs (including 6H (histidine) tags) to express either the parental or one of the six chimeric full-length HAs by infecting insect Sf21 cells. WT-DR, a wild-type (WT) baculovirus expressing only the DsRed fluorescence protein, was also generated as a negative control (FIG. 3A).

Recombinant protein expression was determined by Western blot analysis of infected Sf21 cell lysates, and all recombinant proteins (molecular weight about 70 kDa) could be detected by anti-His antibody. Non-infected cells or cells infected by the WT-DR virus exhibited no expression of HA proteins (FIG. 3B).

Referring to FIGs. 4A to 4D, the structural regions of the chimeric HA proteins containing amino acid residues relevant to chimera functions were defined. FIGs. 4A to 4C illustrated the fusion peptide pocket, the HA1 regions near the spring-loaded long coiled-coil helix of the HA2 subunit, and the HA1-HA1 interface of the FrB chimeric protein, while FIG. 4D illustrated the HA1-HA2 interface of the FrC chimeric protein. Key amino acids contributing to improved HA stability were indicated and labeled on the protein structures as shown in FIGs. 4A to 4D.

The localization of the chimeric HA proteins in the cells was determined by immunofluorescence staining, and it was found that in addition to the two parental HAs, FrB and FrC could also be detected on the insect cell membrane (FIG. 5). Furthermore, upon permeabilizing cells by 0.2% Triton treatment, FrA, FrD, FrE, and FrF chimeric proteins could

be detected inside cells by using the anti-His antibody (FIG. 6).

Example 3: Characterization and bioactivity assessments of parental and chimeric HA proteins

To determine whether the chimeric HA proteins preserved the HA conformation and bioactivity, the recognition by an H7-specific neutralizing monoclonal antibody (11082-R002, Sino Biological Inc., China)[13] of the HA constructs was determined in a cell-based ELISA assay. Since this monoclonal antibody neutralizes infection by an H7N9 influenza virus, it may recognize the viral structural epitope, and thus its reactivity to a chimeric protein indicates that the chimeric HAs are highly likely to preserve the functional HA structure and are more likely to elicit a functional antibody response upon immunization.

Sf21 cell samples membrane-permeabilized by 0.2% Triton treatment revealed that the H7-specific monoclonal antibody recognized FH7 and partially cross-reacted with FH3 (FIG. 7). Further, it was observed that FrB and FrC were recognized by this antibody to a degree comparable to FH7 (FIG. 7).

Moreover, the hemagglutination activity (a key feature of the HA protein) of the HA constructs was determined. First, Sf21 cells infected by recombinant viruses were disrupted by brief sonication to expose the cytosolic HAs. The disrupted cell suspensions were then serially 2-fold diluted and mixed with turkey red blood cells. If functional trimeric HAs exist in the disrupted cell suspensions, they would bind to the sialic acid receptors on the surfaces of the red blood cells and form clumps of red blood cell lattices[14, 15] (FIG. 8A). It was found that in addition to the cells expressing FrB, FrC could also agglutinate turkey red blood cells (FIG. 8B). These results suggest that FrB and FrC preserved the conformation and function of HA after recombination.

Example 4: Assay of thermal stability

To analyze the thermal stability of cell-expressed HAs, thermal hemagglutination assay protocols from other literature[8, 16] were adopted, which use loss of hemagglutination titer (HA titer) during heating to evaluate the thermal stability of HA proteins.

HA titers were initially determined for the infected cells, and then the cell amounts were adjusted to an HA titer of 64. The cells were incubated at 50°C for different time periods and then cooled down to 4°C for the hemagglutination assay.

It was found that FH7 exhibited gradual loss of HA titer immediately upon starting the heating process and had completely lost its hemagglutination activity after 20 min of heating. The other parental sample, FH3, showed a gradual decrease of the hemagglutination activity for the initial 30 min of heating, but it retained the HA titer until the end of the 120-min experimental period. For cells expressing either FrB or FrC, HA titers decreased during the initial 10 to 20 min of heating but then maintained near constant titers toward the end of the heating process. Cells infected by WT-DR were used as a negative control and showed no HA titer during the experimental period (FIG. 9). These results suggest that the FrB and FrC proteins exhibit significantly enhanced stability at 50°C compared to the parental FH7 protein.

Example 5: Assay of eliciting neutralizing antibodies against the H7N9 virus

To explore if the chimeric HA proteins could still serve as efficient immunogens for triggering neutralizing antibodies against the H7N9 virus, the FH7, FrB, and FrC proteins were extracted from the infected insect cells to immunize mice, and their immune responses were further analyzed.

Three groups of five female BALB/c mice were immunized intraperitoneally with 30 μg of purified FH7, FrB, or FrC proteins, respectively. As negative controls, five mice were

injected with PBS alone. Each mouse received two booster shots at week 2 and week 4 after primary immunization, and then the blood samples were collected at week 6 and week 8. The serum H7-specific IgG levels were determined by indirect ELISA using purified FH7 as an antigen (FIG. 10A). Mice immunized with FH7 protein showed a significantly higher H7-specific IgG antibody response on both week 6 and week 8 compared to the group immunized with PBS alone. Similarly, the groups immunized with either FrB or FrC showed levels of the H7-specific IgG response comparable to the FH7 group at these two time points (FIG. 10A).

Further, a microneutralization assay was conducted to determine whether the immunized sera can neutralize real H7N9 influenza virus infection (FIG. 10B). H7N9 influenza viruses (the A/Taiwan/01/2013 strain) was incubated with serially diluted mouse sera, which were then used to infect Madin-Darby canine kidney (MDCK) cells. The microneutralization titer was determined at 3 d.p.i. as the reciprocal of the highest dilution without a virus-induced cytopathic effect (CPE). Sera from mice immunized with FrB or FrC presented a higher microneutralization titer compared to FH7-immunized sera at week 6 and a comparable titer at week 8. These data indicate that the chimeric HA proteins can elicit H7-specific antibodies that are able to inhibit H7N9 viral infection.

From the above, it can be seen that the recombinant chimeric proteins of the present disclosure generated from different influenza viruses by non-contiguous SCHEMA recombination have enhanced thermal stability, while maintaining proper antigenicity and high neutralizing efficiency.

It is known that homology of the parental proteins used in a SCHEMA approach affects the number of functional chimeras that can be derived. Nevertheless, even though the H7-HA1 and H3-HA1 sequences used in the present disclosure share only 38% sequence identity, the

chimeric HA proteins are still expressed (FIG. 3B) and exhibit authentic HA function (i.e., sialic acid receptor binding), as assayed by the hemagglutination assay (FIG. 8B), implying that chimeric HA proteins can serve as better vaccine antigens to tackle H7N9 viruses.

Further, since the chimeric HA proteins of the present disclosure exhibit much higher thermal stability than FH7, they are more likely to support long-term storage and transportation as vaccine products.

While some of the embodiments of the present disclosure have been described in detail above, it is, however, possible for those of ordinary skill in the art to make various modifications and changes to the particular embodiments shown without substantially departing from the teaching and advantages of the present disclosure. Such modifications and changes are encompassed in the scope of the present disclosure as set forth in the appended claims.

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# Claims

What is claimed is:

1. A chimeric hemagglutinin (HA) protein comprising an HA1 subunit and an HA2 subunit, wherein the HA1 subunit is composed of a first domain derived from a parental HA1 subunit of a first subtype influenza virus and a second domain derived from a parental HA1 subunit of a second subtype influenza virus.

2. The chimeric HA protein according to claim 1, wherein the first subtype influenza virus and the second subtype influenza virus are independently selected from the group consisting of H1 to H18 subtype influenza viruses, provided that the first subtype influenza virus and the second subtype influenza virus are different.

3. The chimeric HA protein according to claim 1, wherein the HA1 subunit has an amino acid identity less than 100% as compared with the parental HA1 subunit of the first subtype influenza virus.

4. The chimeric HA protein according to claim 1, wherein the HA1 subunit has an amino acid identity of at least 30% as compared with the parental HA1 subunit of the first subtype influenza virus.

5. The chimeric HA protein according to claim 1, wherein the HA1 subunit has an amino acid identity of between 70% and 95% as compared with the parental HA1 subunit of the first subtype influenza virus.

6. The chimeric HA protein according to claim 5, wherein the amino acid identity of the HA1 subunit compared to the parental HA1 subunit of the first subtype influenza virus is between 88% and 91%.

7. The chimeric HA protein according to claim 1, wherein the second domain is at least one portion of an HA structural region selected from the group consisting of a fusion peptide pocket, an HA1 region near a spring-loaded long coiled-coil helix of the HA2 subunit, an HA1-HA1 interface, and an HA1-HA2 interface.

8. The chimeric HA protein according to claim 7, comprising at least one of:

(1) the fusion peptide pocket including Ala, Thr, Leu, Asn, Lys, and Arg;

(2) the HA1 region near the spring-loaded long coiled-coil helix of the HA2 subunit, the HA1 region including Asp and Ser;

(3) the HA1-HA1 interface including Asn and Ser; and

(4) the HA1-HA2 interface including Arg, Val, Lys, Ile, Tyr, and Ala.

9. The chimeric HA protein according to claim 1, wherein the first subtype influenza virus is an H7 subtype influenza virus.

10. The chimeric HA protein according to claim 1, wherein the second subtype influenza virus is an H3 subtype influenza virus.

12. The chimeric HA protein according to claim 1, wherein the parental HA1 subunit of the second subtype influenza virus has an amino acid sequence of SEQ ID NO: 2, and the second

domain is derived from at least one amino acid, at least one peptide or a combination thereof selected from the group consisting of positions #11-#13, #21, #25, #27, #29, #31-#34, #37, #42, #44-#45, #46-#50, #53-#56, #58, #185-#189, #193, #216-#217, #219, #228, #268-#269, #271-#274, #276, #278-#280, #282-#285, #287, #289-#292, #297-#302, #304, #307, #312-#313, #315, #321, and #326-#329 of SEQ ID NO: 2.

13. The chimeric HA protein according to claim 1, wherein the HA1 subunit comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 8.

14. The chimeric HA protein according to claim 1, wherein the HA2 subunit is an HA2 subunit of the first subtype influenza virus.

15. A vaccine composition comprising the chimeric HA protein of claim 1 and a pharmaceutically acceptable carrier thereof, wherein the chimeric HA protein is present in an amount effective in preventing influenza virus infection.

16. The vaccine composition according to claim 15, further comprising an adjuvant.

17. A method of inducing an immune response against an influenza virus in a subject in need thereof, comprising administering the vaccine composition according to claim 15 to the subject.

18. The method according to claim 17, wherein the influenza virus is H1N1, H1N2, H2N2, H3N2, H5N1, H5N2, H5N6, H6N1, H7N2, H7N3, H7N7, H7N9, H9N2, H10N7 or H10N8 influenza virus.

19. The method according to claim 17, wherein the HA1 subunit of the chimeric HA protein has an amino acid identity of at least 30% and less than 100% as compared with an HA1 subunit of the influenza virus.

5

20. The method according to claim 19, wherein the HA1 subunit of the chimeric HA protein comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 3 to 8.

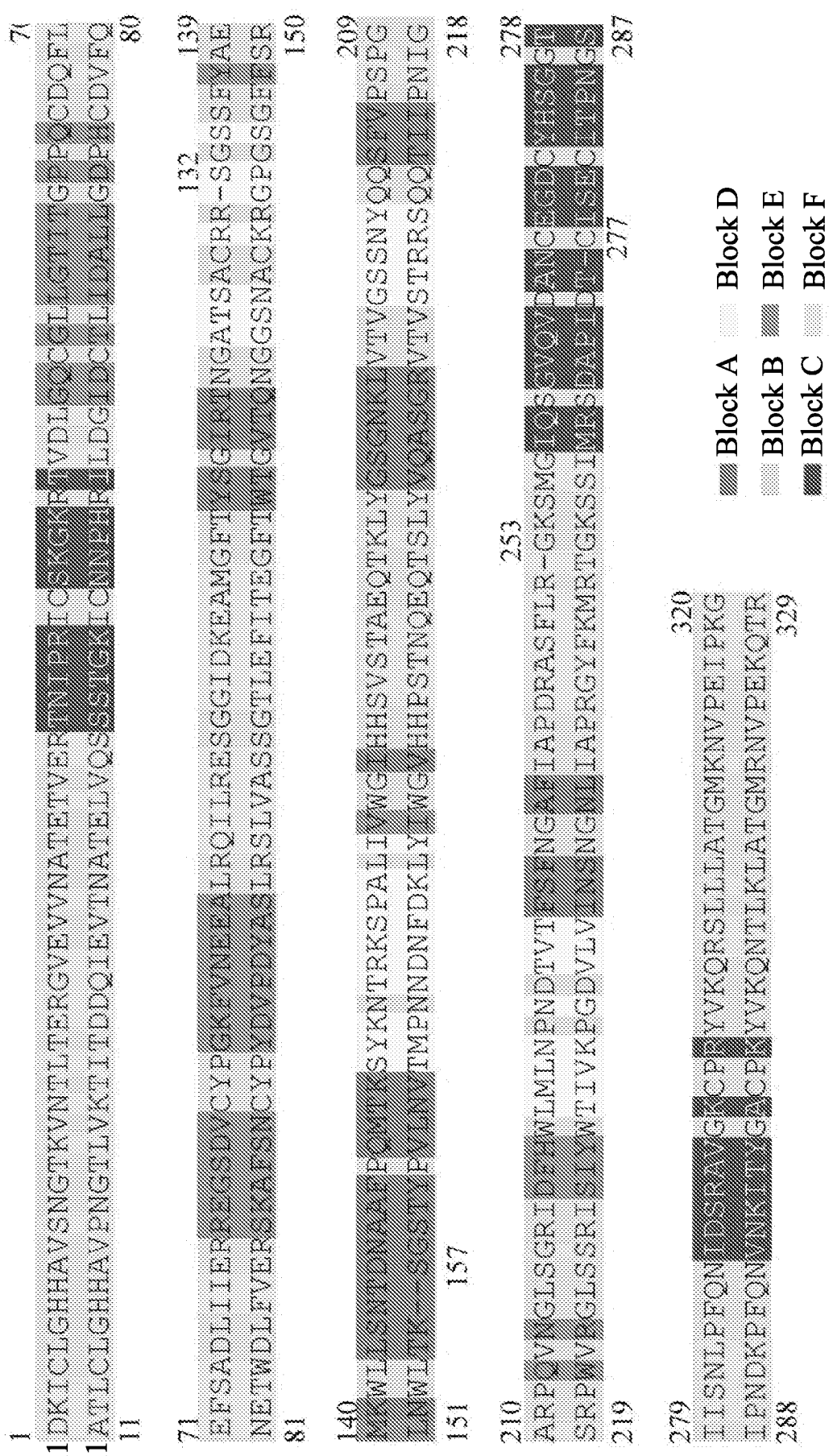



FIG. 1A

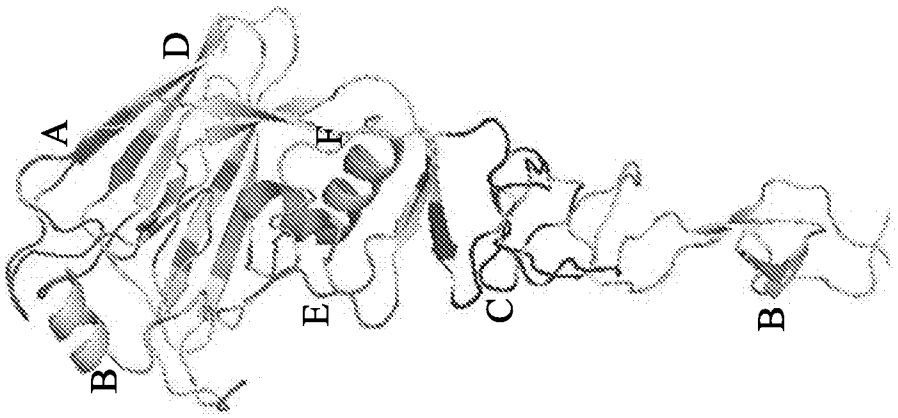



FIG. 1B

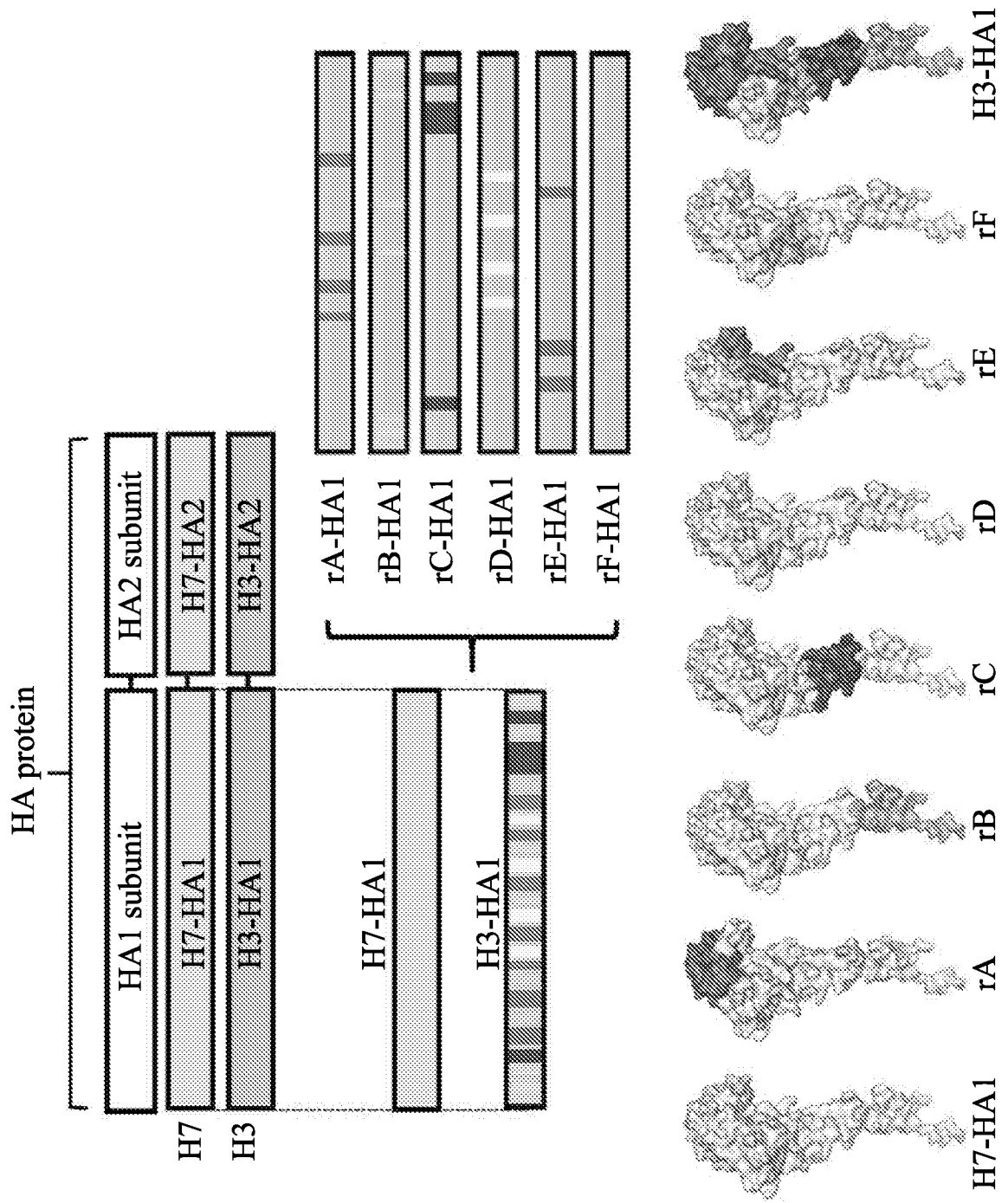

HA protein
HA1 subunit
HA2 subunit
H7
H3
H7-HA1
H3-HA1
H7-HA2
H3-HA2
H7-HA1
H3-HA1
rA-HA1
rB-HA1
rC-HA1
rD-HA1
rE-HA1
rF-HA1
H7-HA1
rA
rB
rC
rD
rE
rF
H3-HA1

FIG. 2

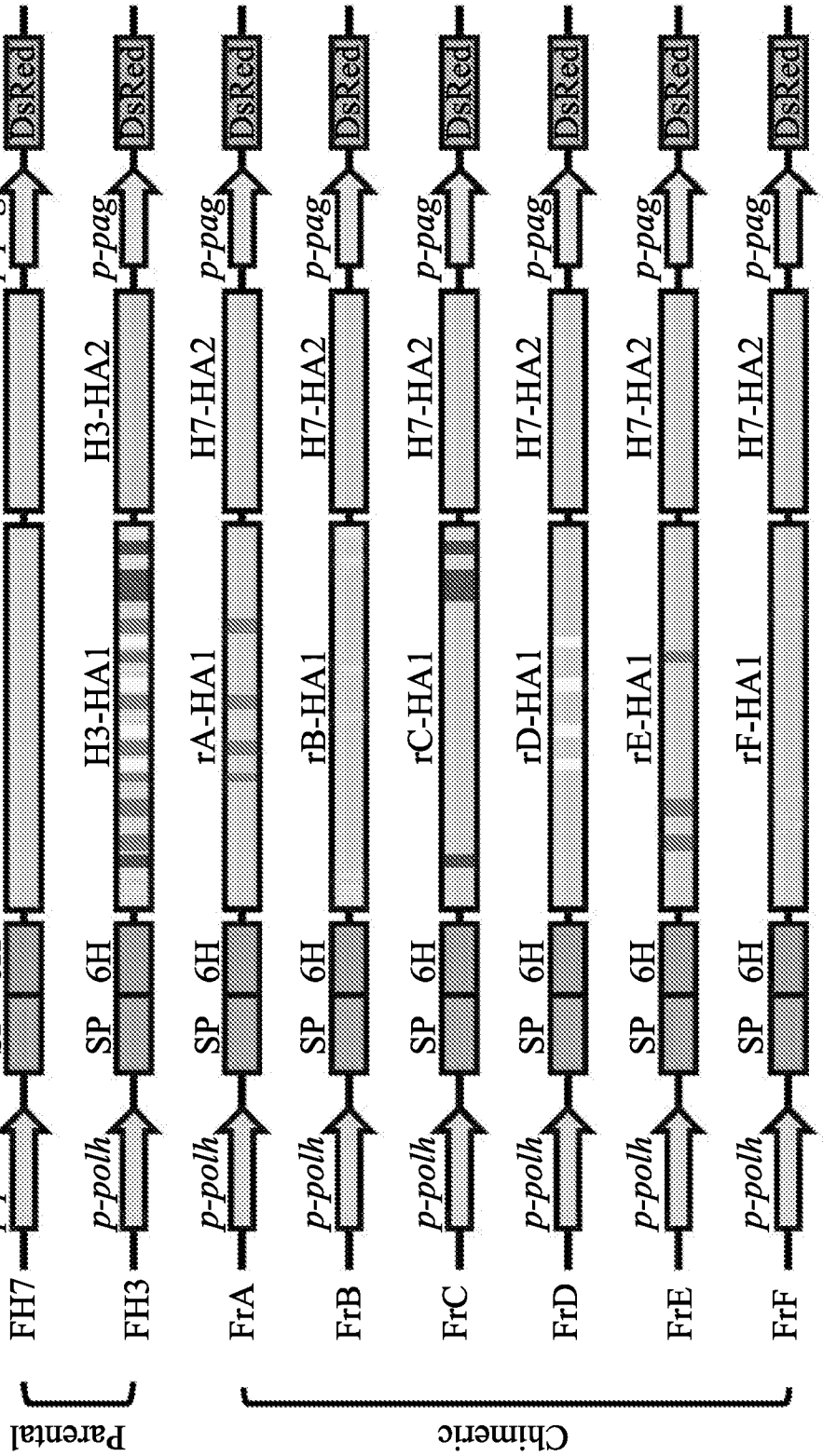

Parental
Chimeric
FH7
FH3
FrA
FrB
FrC
FrD
FrE
FrF
WT-DR
p-polh
SP
6H
H7-HA1
H3-HA1
rA-HA1
rB-HA1
rC-HA1
rD-HA1
rE-HA1
rF-HA1
H7-HA2
H3-HA2
p-pag
DsRed

FIG. 3A

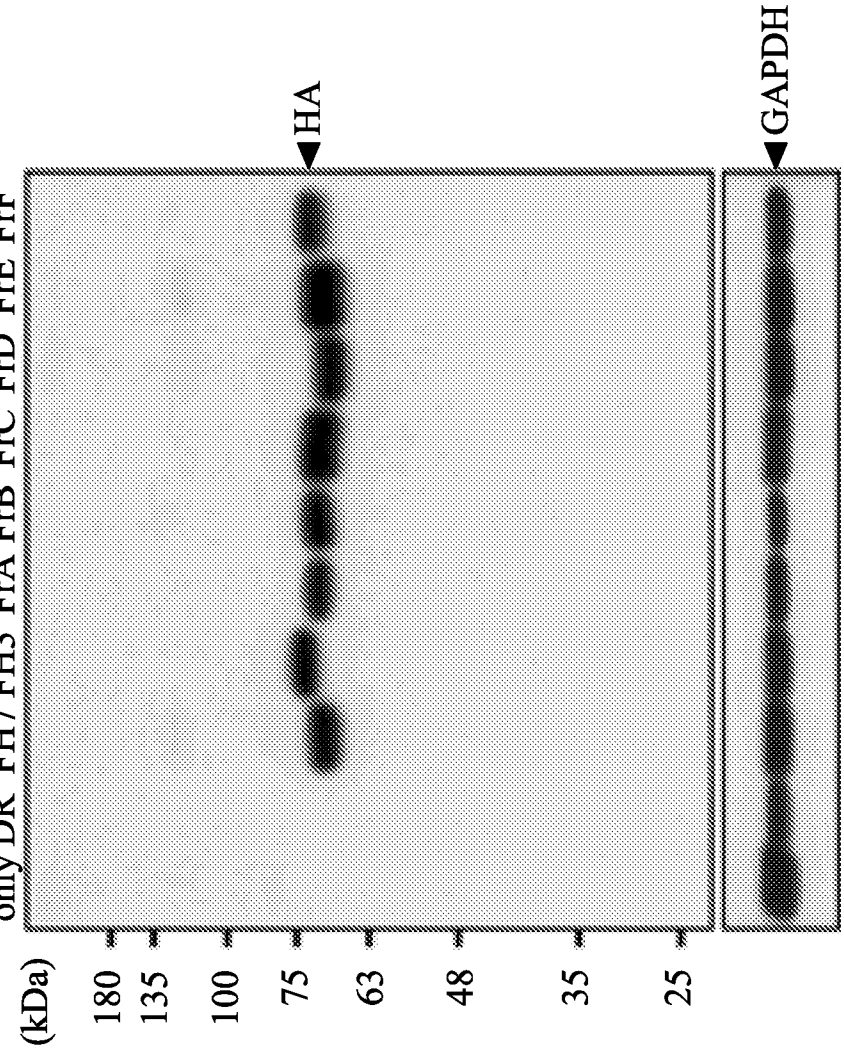

Cell only
WT-DR
Parental
FH7
FH3
Chimeric
FrA
FrB
FrC
FrD
FrE
FrF
(kDa)
180
135
100
75
63
48
35
25
HA
GAPDH

FIG. 3B

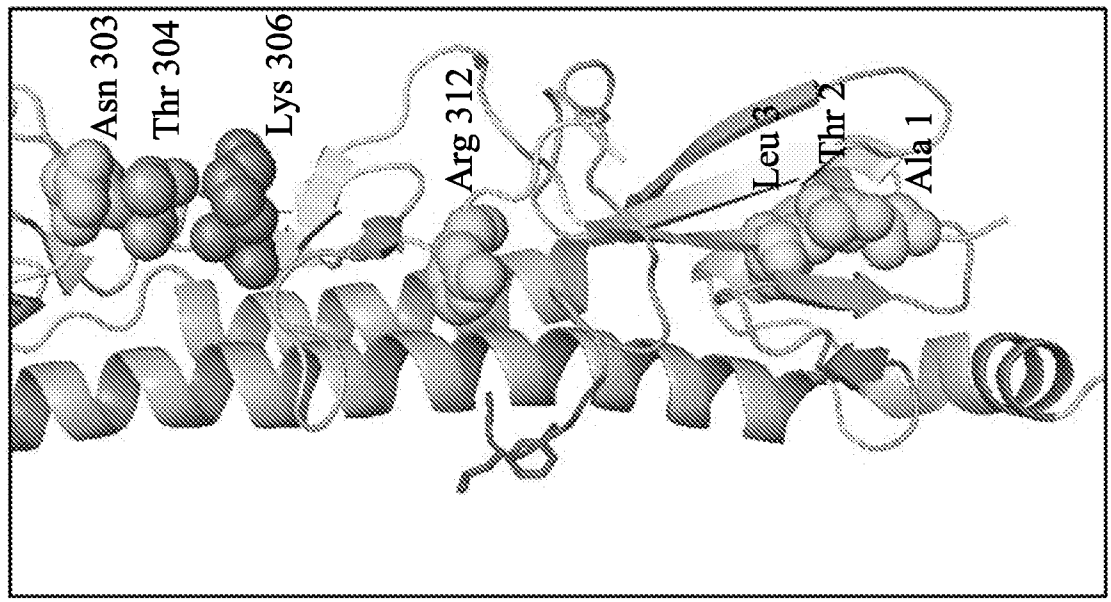



FIG. 4A

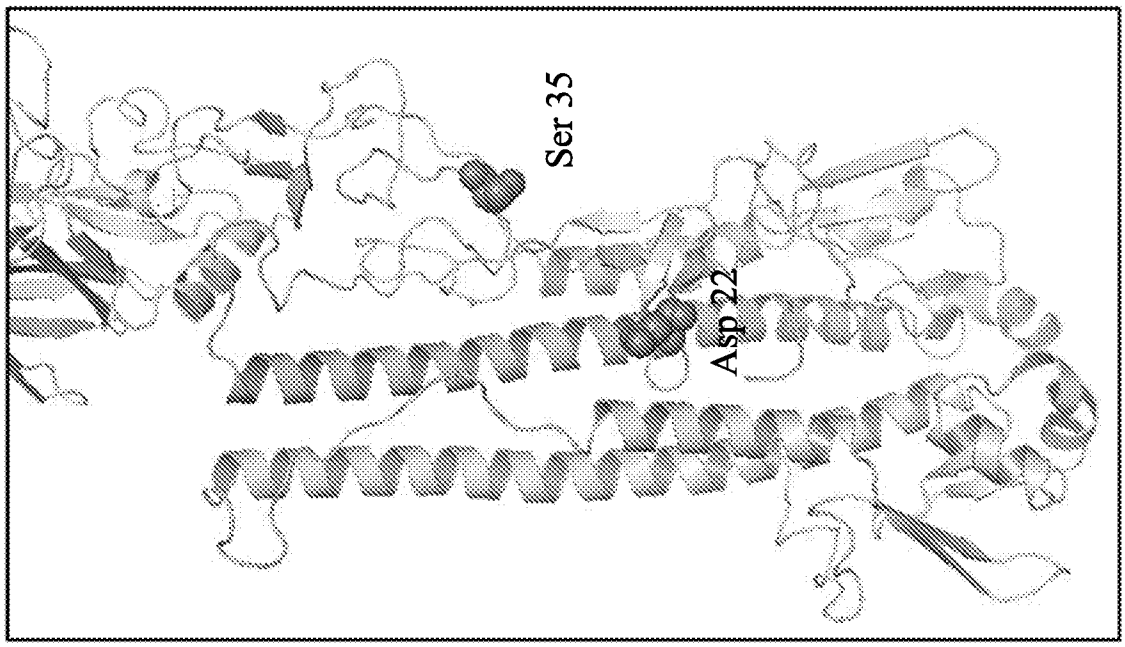



FIG. 4B

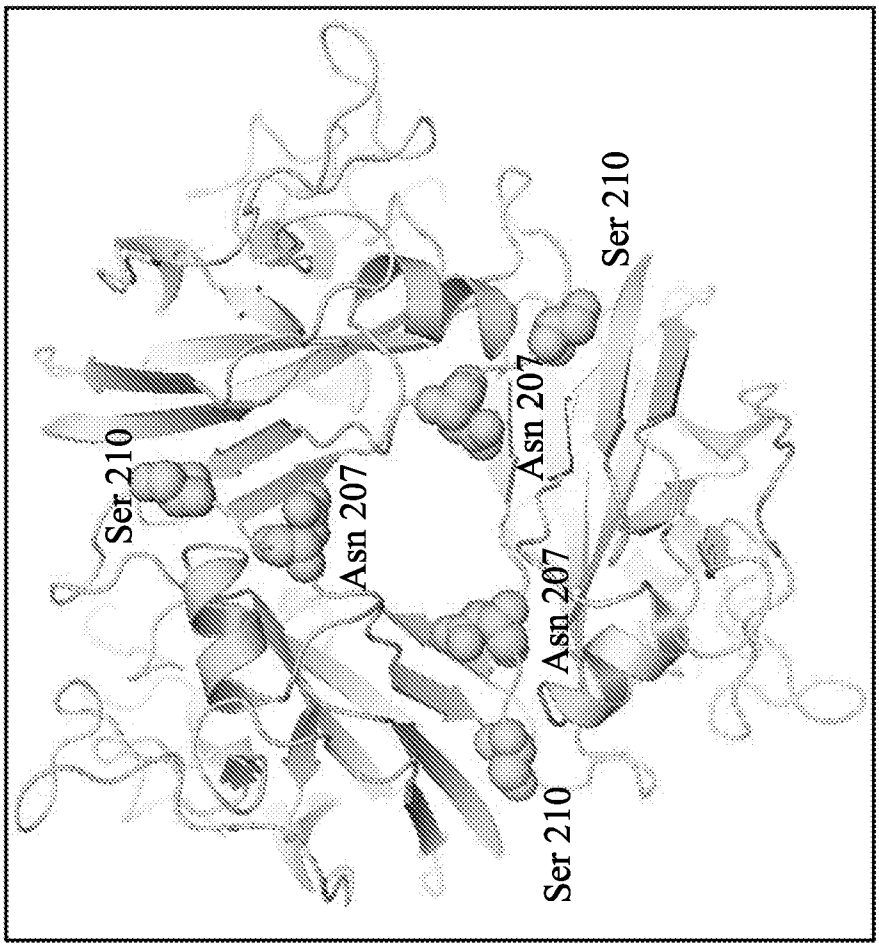



FIG. 4C

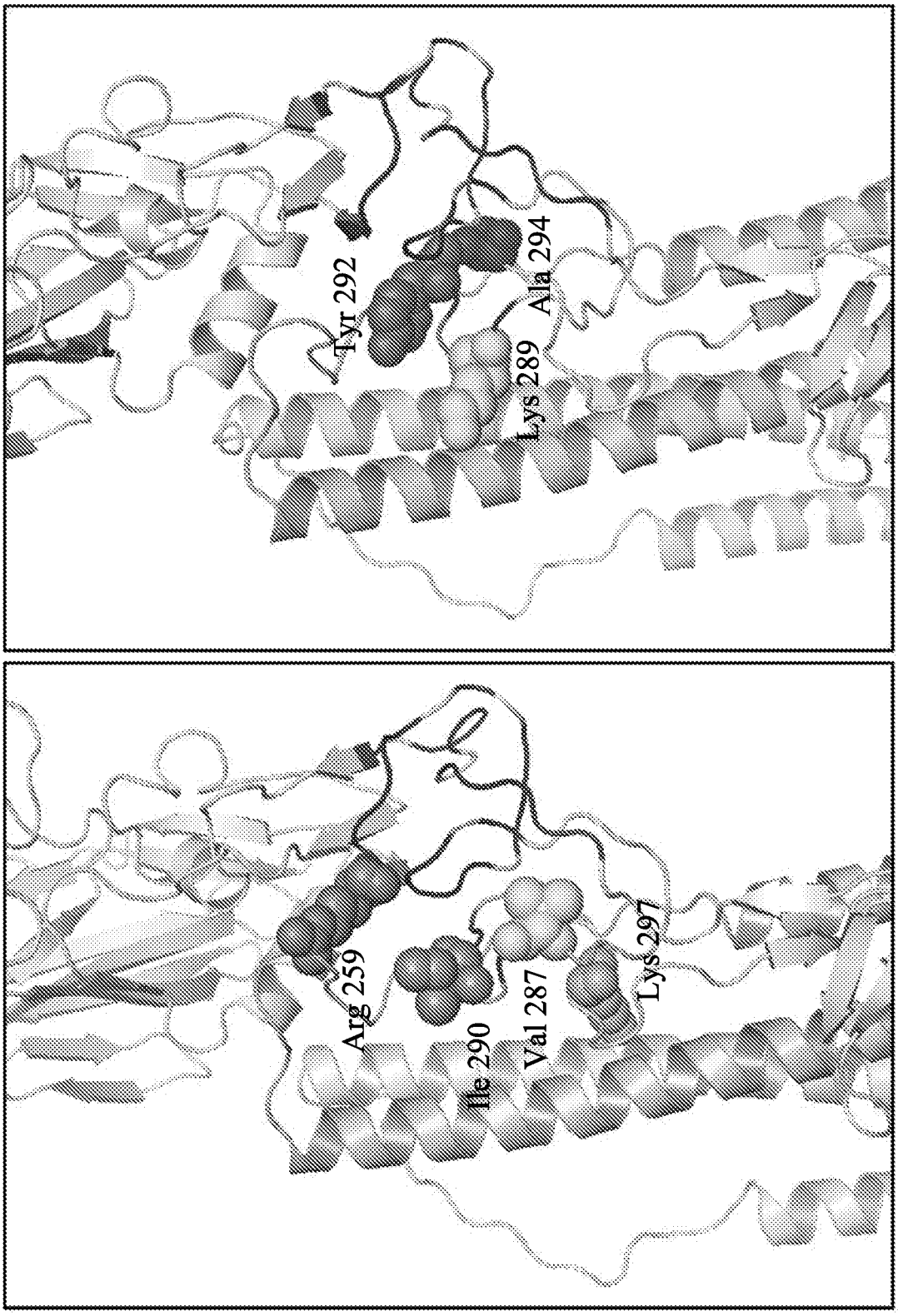



FIG. 4D

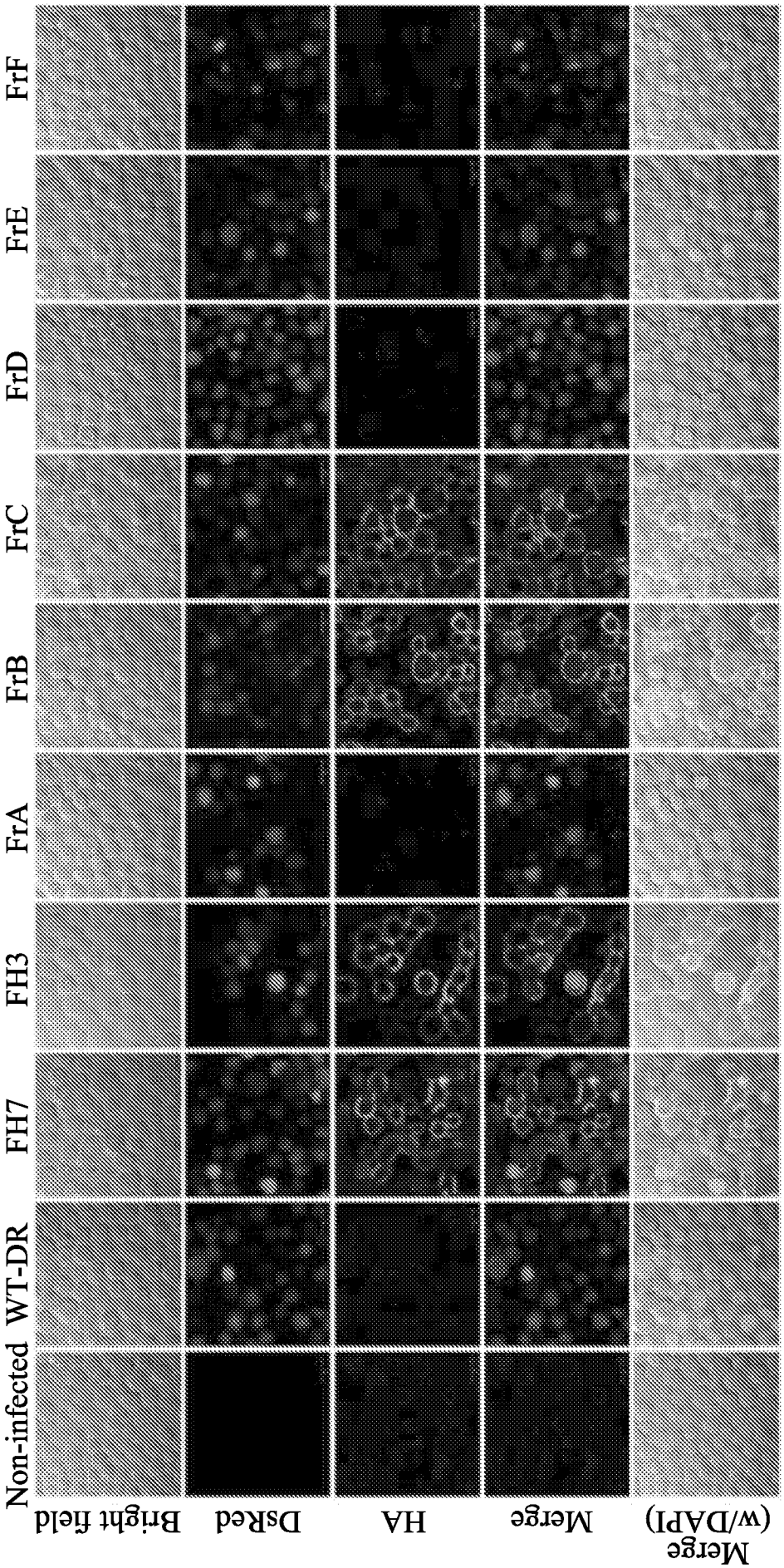

Non-infected
WT-DR
FH7
FH3
FrA
FrB
FrC
FrD
FrE
FrF
Bright field
DsRed
HA
Merge
Merge (w/DAPI)

FIG. 5

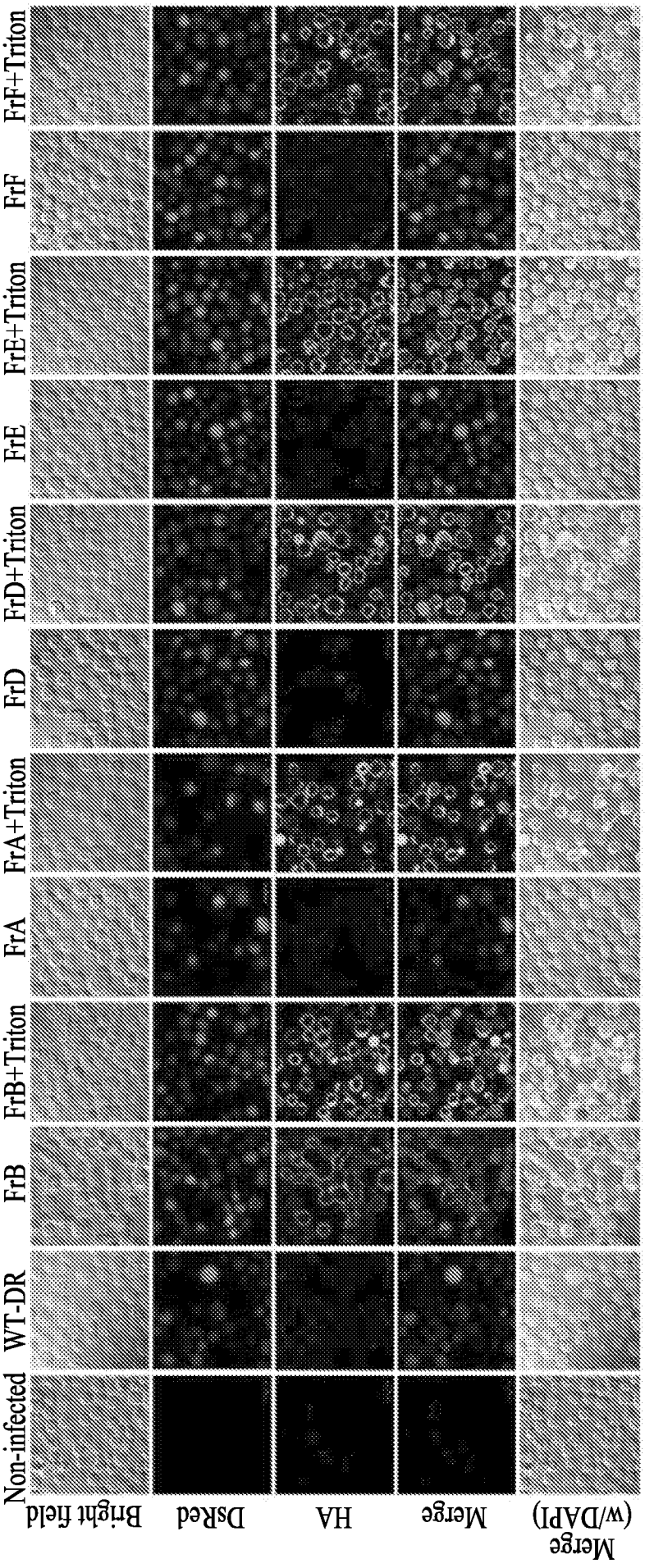

Non-infected
WT-DR
FrB
FrB+Triton
FrA
FrA+Triton
FrD
FrD+Triton
FrE
FrE+Triton
FrF
FrF+Triton
Bright field
DsRed
HA
Merge
Merge (w/DAPI)

FIG. 6

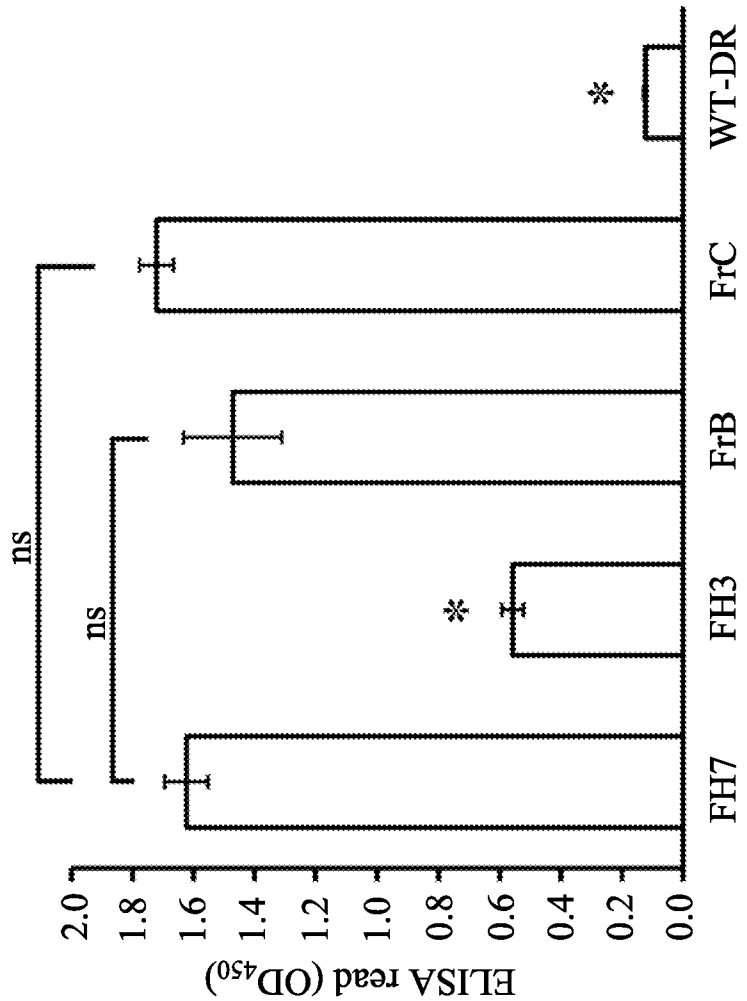

ELISA read ($OD_{450}$)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
FH7
FH3
FrB
FrC
WT-DR
ns
ns
*
*

FIG. 7

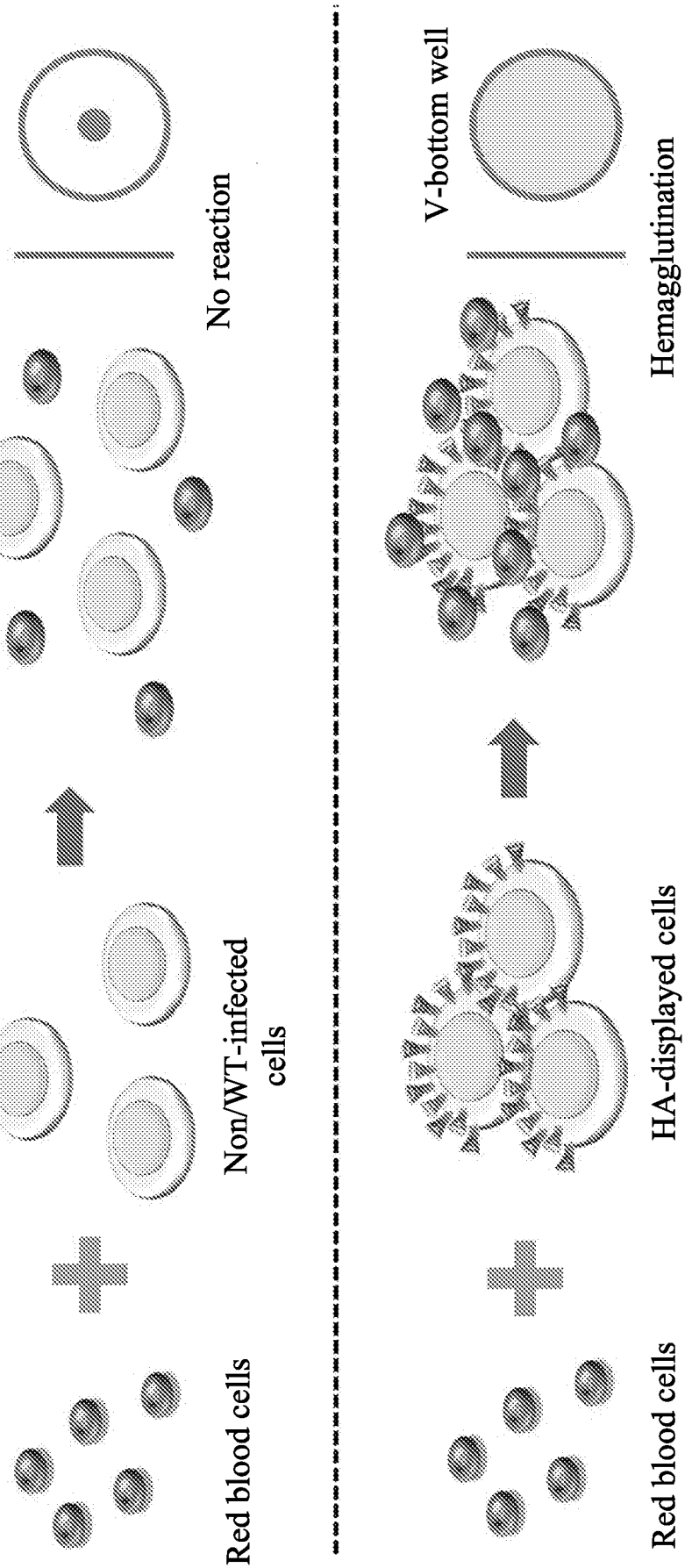

V-bottom well
No reaction
Non/WT-infected cells
Red blood cells
V-bottom well
Hemagglutination
HA-displayed cells
Red blood cells

FIG. 8A

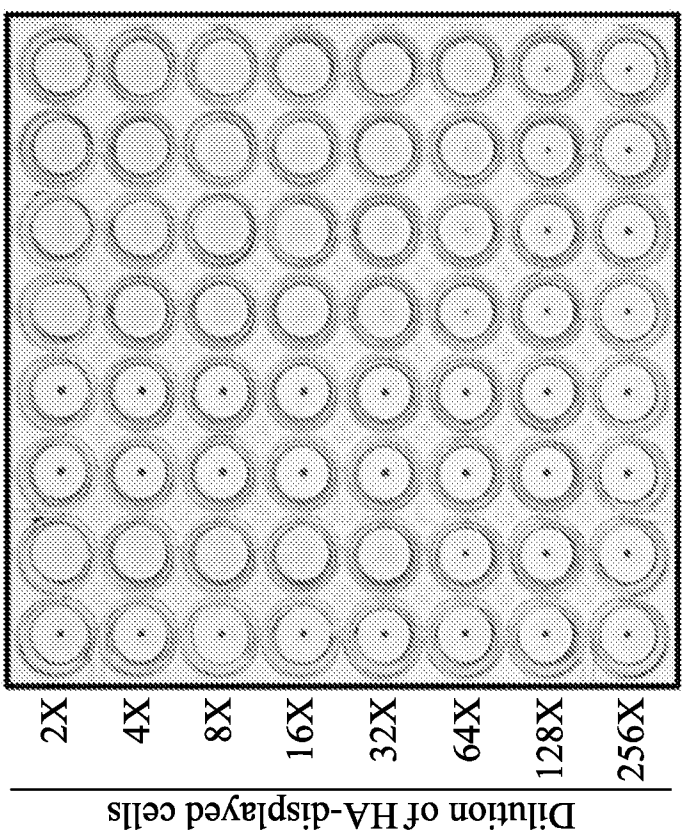

PBS
HA
Non
WT-DR
Parental
FH7
FH3
Chimeric
FrB
FrC
2X
4X
8X
16X
32X
64X
128X
256X
Dilution of HA-displayed cells

FIG. 8B

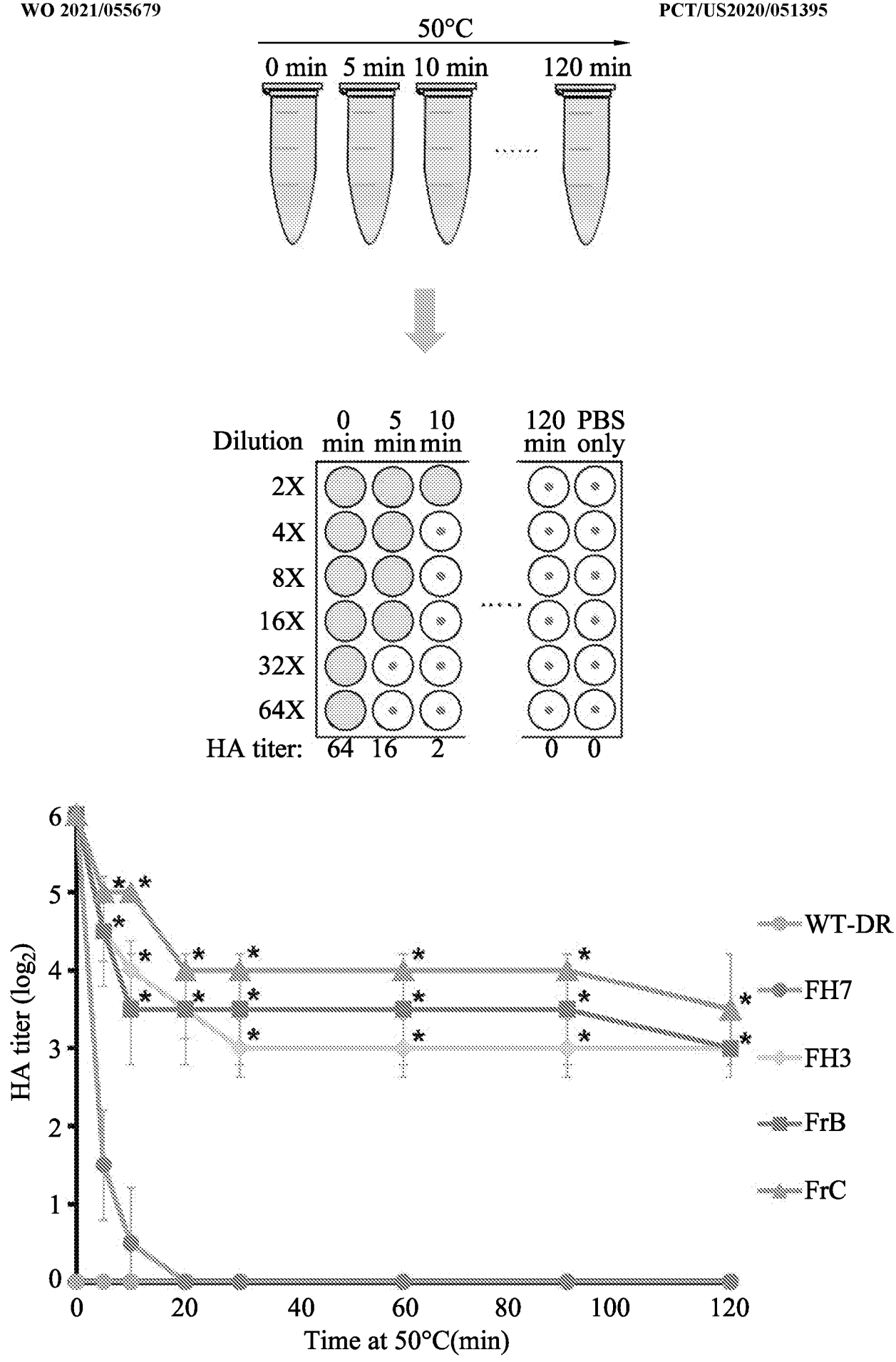

50°C
0 min
5 min
10 min
120 min
Dilution
0 min
5 min
10 min
120 min
PBS only
2X
4X
8X
16X
32X
64X
HA titer:
64
16
2
0
0
HA titer (log2)
Time at 50°C(min)
WT-DR
FH7
FH3
FrB
FrC

FIG. 9

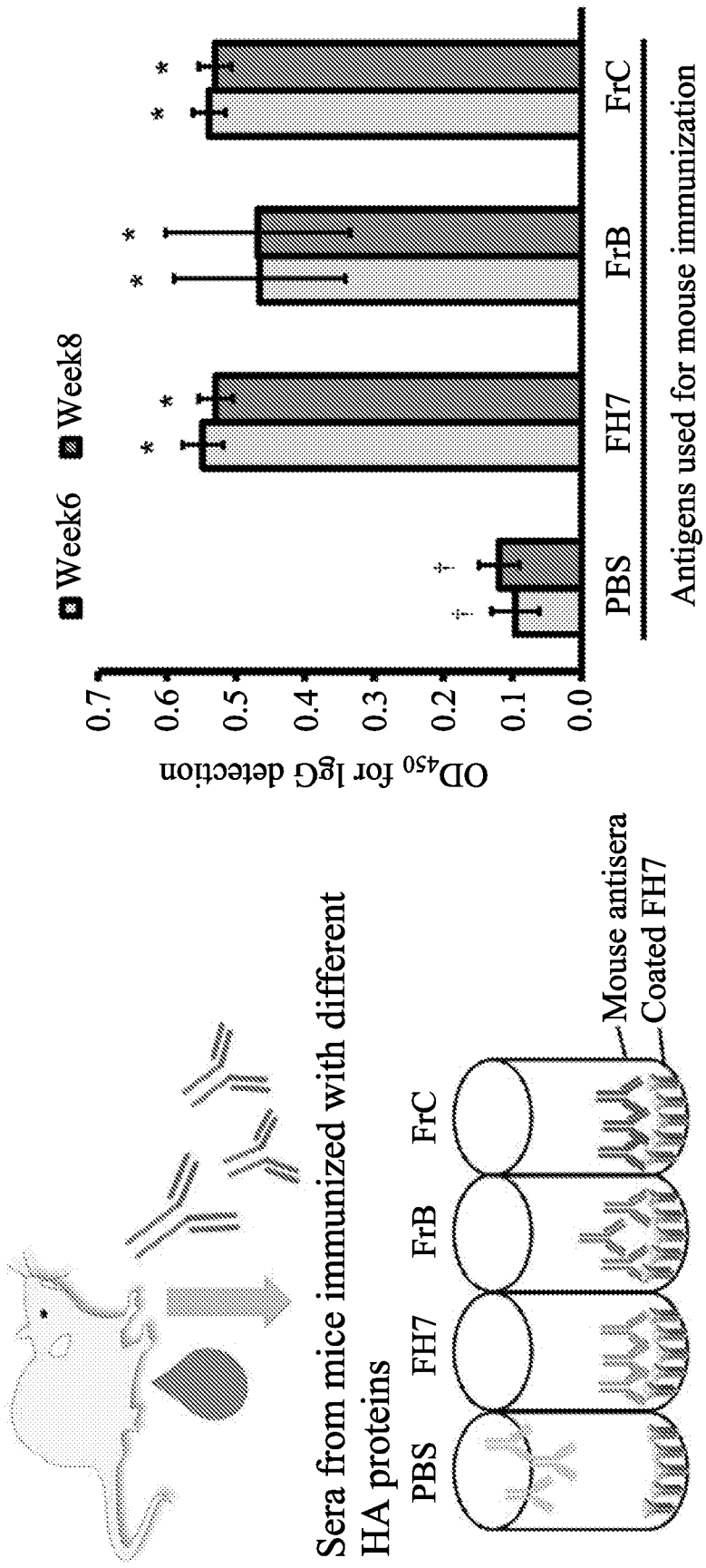
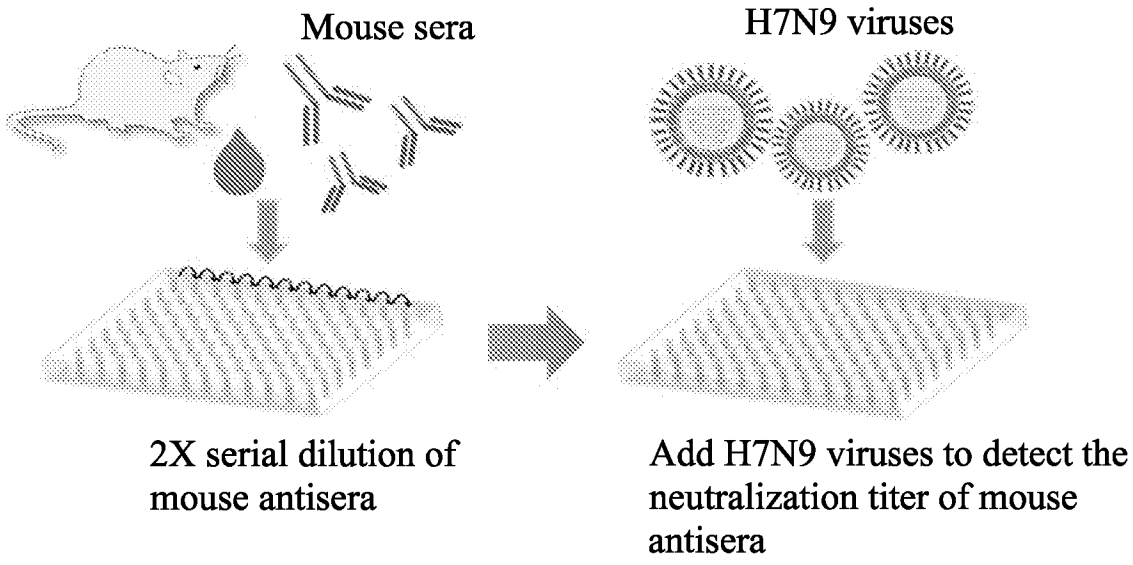

Sera from mice immunized with different HA proteins
PBS
FH7
FrB
FrC
Mouse antisera
Coated FH7
OD450 for IgG detection
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Week6
Week8
PBS
FH7
FrB
FrC
Antigens used for mouse immunization

FIG. 10A


Mouse sera
H7N9 viruses
2X serial dilution of mouse antisera
Add H7N9 viruses to detect the neutralization titer of mouse antisera

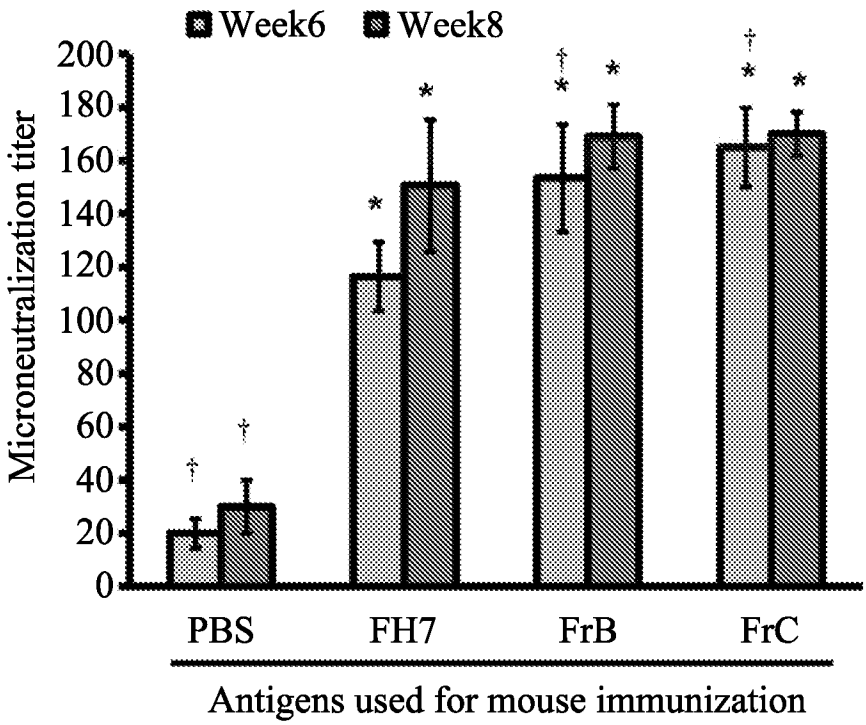

Week6
Week8
Microneutralization titer
200
180
160
140
120
100
80
60
40
20
0
PBS
FH7
FrB
FrC
Antigens used for mouse immunization

FIG. 10B

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT/US2020/051395**

**A. CLASSIFICATION OF SUBJECT MATTER**

**C07K 14/005(2006.01)i, A61K 39/145(2006.01)i, A61P 31/16(2006.01)i**

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)
C07K 14/005; A61K 39/145; A61K 39/395; C07K 14/11; C07K 16/08; C07K 16/10; C12N 1/15; C12N 15/09; G01N 33/569; A61P 31/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: chimeric hemagglutinin (HA), influenza virus, chimeric subunit, vaccine, h7-ha1, h3-ha1

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KR 10-1835989 B1 (CHUNGBUK NATIONAL UNIVERSITY INDUSTRY-ACADEMIC COOPERATION FOUNDATION et al.) 08 March 2018<br>abstract; claim 1; paragraphs [0005]-[0023] | 1-6,9,10,14-19 |
| A | | 7,8,12,13,20 |
| A | US 2013-0309248 A1 (CRUCELL HOLLAND B.V.) 21 November 2013<br>claims 1-29 | 1-10,12-20 |
| A | US 2008-0014205 A1 (HOROWITZ, LAWRENCE et al.) 17 January 2008<br>claims 1-93 | 1-10,12-20 |
| A | US 10272148 B2 (COUTURE, MANON et al.) 30 April 2019<br>claims 1-17 | 1-10,12-20 |
| A | JP 2014-523254 A (INSTITUTE FOR RESEARCH IN BIOMEDICINE) 11 September 2014<br>claims 1-35 | 1-10,12-20 |
| PX | TSAI, CHIH-HSUAN et al., `Generation of stable influenza virus hemagglutinin through structure-guided recombination`,<br>ACS Synthetic Biology, 30 September 2019 (Online publication date), Vol. 8,<br>pages 2472-2482<br>pages 2472-2479; and figures 1-9 | 1-10,12-20 |

☐ 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 | Date of mailing of the international search report |
|---|---|
| 04 January 2021 (04.01.2021) | **06 January 2021 (06.01.2021)** |

Name and mailing address of the ISA/KR
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Korean Intellectual Property Office
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Authorized officer
HEO, Joo Hyung
Telephone No. +82-42-481-5373

# INTERNATIONAL SEARCH REPORT

International application No.
**PCT/US2020/051395**

**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.: 11
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:
Claim 11 does not comply with PCT Rule 6.1(b), because claim 11 is missing in this application.

3. ☐ Claims Nos.:
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 addtional 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 an additional fees, this Authority did not invite payment of any 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.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2020/051395**

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| KR 10-1835989 B1 | 08/03/2018 | None | |
| US 2013-0309248 A1 | 21/11/2013 | AR 076570 A1 | 22/06/2011 |
| | | AU 2010-247530 A1 | 03/11/2011 |
| | | AU 2010-247530 A1 | 18/11/2010 |
| | | AU 2010-247530 B2 | 13/10/2016 |
| | | CA 2761648 A1 | 18/11/2010 |
| | | CA 2761648 C | 12/03/2019 |
| | | CN 102448986 A | 09/05/2012 |
| | | CN 102448986 B | 25/11/2015 |
| | | CN 105418757 A | 23/03/2016 |
| | | CN 105418757 B | 09/07/2019 |
| | | CU 20110206 A7 | 15/10/2012 |
| | | CU 23938 B1 | 29/08/2013 |
| | | EA 029939 B1 | 29/06/2018 |
| | | EA 201171383 A1 | 30/05/2012 |
| | | EP 2430046 A1 | 21/03/2012 |
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