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(54) Title: STABILIZED PRE-FUSION RSV F PROTEINS

(57) Abstract: The present invention provides stable pre-fusion respiratory syncytial virus (RSV) F proteins, immunogenic compositions comprising said proteins and uses thereof for the prevention and/or treatment of RSV infection.

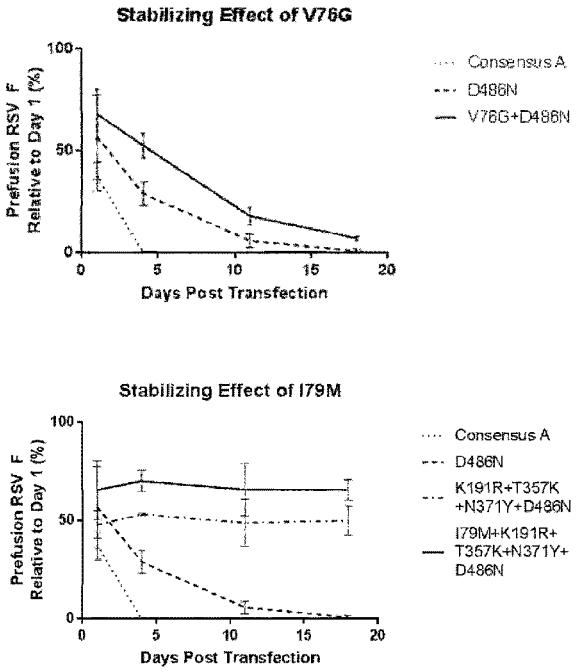


Fig. 3

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# STABILIZED PRE-FUSION RSV F PROTEINS

The present invention relates to the field of medicine. The invention in particular relates to recombinant pre-fusion RSV F proteins, to nucleic acid molecules encoding the RSV F proteins, and uses thereof, e.g. in vaccines.

## BACKGROUND OF THE INVENTION

After discovery of the respiratory syncytial virus (RSV) in the 1950s, the virus soon became a recognized pathogen associated with lower and upper respiratory tract infections in humans. Worldwide, it is estimated that 64 million RSV infections occur each year resulting in 160.000 deaths (WHO Acute Respiratory Infections Update September 2009). The most severe disease occurs particularly in premature infants, the elderly and immunocompromised individuals. In children younger than 2 years, RSV is the most common respiratory tract pathogen, accounting for approximately 50% of the hospitalizations due to respiratory infections, with the peak of hospitalization occurring at 2-4 months of age. It has been reported that almost all children have been infected by RSV by the age of two. Repeated infection during lifetime is attributed to ineffective natural immunity. In the elderly, the RSV disease burden is similar to that caused by non-pandemic influenza A infections.

RSV is a paramyxovirus, belonging to the subfamily of *Pneumoviridae*. Its genome encodes for various proteins, including the membrane proteins known as RSV Glycoprotein (G) and RSV fusion (F) protein which are the major antigenic targets for neutralizing antibodies. Antibodies against the fusion-mediating part of the F1 protein can prevent virus uptake in the cell and thus have a neutralizing effect.

RSV F fuses the viral and host-cell membranes by irreversible protein refolding from the labile pre-fusion conformation to the stable post-fusion conformation. Structures of both conformations have been determined for RSV F (McLellan JS, *et al.* (2010, 2013, 2013);

Swanson KA, *et al.* (2011)), as well as for the fusion proteins from related paramyxoviruses, providing insight into the complex mechanism of this fusion protein. Like other class I fusion proteins, the inactive precursor, RSV $F_0$, requires cleavage during intracellular maturation by a furin-like protease. RSV F contains two furin cleavage sites, which leads to three proteins: F2, p27 and F1, with the latter containing a hydrophobic fusion peptide (FP) at its N-terminus. In order to refold from the pre-fusion to the post-fusion conformation, the refolding region 1 (RR1) between residue 137 and 216, that includes the FP and heptad repeat A (HRA) has to transform from an assembly of helices, loops and strands to a long continuous helix. The FP, located at the N-terminal segment of RR1, is then able to extend away from the viral membrane and to insert into the proximal membrane of the target cell. Next, the refolding region 2 (RR2), which forms the C-terminal stem in the pre-fusion F spike and includes the heptad repeat B (HRB), relocates to the other side of the RSV F head and binds the HRA coiled-coil trimer with the HRB domain to form the six-helix bundle. The formation of the RR1 coiled-coil and relocation of RR2 to complete the six-helix bundle are the most dramatic structural changes that occur during the refolding process.

A vaccine against RSV infection is currently not yet available, but it is highly desired due to the high disease burden. The RSV fusion glycoprotein (RSV F) is an attractive vaccine antigen as it is the principal target of neutralizing antibodies in human sera. Most neutralizing antibodies in human sera are directed against the pre-fusion conformation, but due to its instability the pre-fusion conformation has a propensity to prematurely refold into the post-fusion conformation, both in solution and on the surface of the virions. As indicated above, crystal structures have revealed a large conformational change between the pre-fusion and post-fusion states. The magnitude of the rearrangement suggested that only a portion of antibodies directed to the post-fusion conformation of RSV-F will be able to cross react with the native conformation of the pre-fusion spike on the surface of the virus. Accordingly,

efforts to produce a vaccine against RSV have focused on developing vaccines that contain pre-fusion forms of RSV F protein (see, e.g., WO20101149745, WO2010/1149743, WO2009/1079796, WO2012/158613) but until this date, still no vaccine is available.

Therefore, a need remains for efficient vaccines against RSV, in particular vaccines comprising RSV F proteins in the pre-fusion conformation. The present invention aims at providing means for obtaining such stable pre-fusion RSV F proteins for use in vaccinating against RSV.

## SUMMARY OF THE INVENTION

The present invention provides stable, recombinant, pre-fusion respiratory syncytial virus (RSV) fusion (F) proteins, i.e. recombinant RSV F proteins that are stabilized in the pre-fusion conformation, and fragments thereof. The pre-fusion RSV F proteins, or fragments thereof, comprise at least one epitope that is specific to the pre-fusion conformation F protein, e.g. as determined by specific binding of an antibody that is specific for the pre-fusion conformation to the proteins. In certain embodiments, the pre-fusion RSV F proteins are soluble multimeric, e.g. trimeric, proteins. The invention also provides nucleic acid molecules encoding the pre-fusion RSV F proteins, or fragments thereof, as well as vectors, e.g. adenovectors, comprising such nucleic acid molecules.

The invention also relates to methods of stabilizing RSV F proteins in the pre-fusion conformation, and to the pre-fusion RSV F proteins obtainable by said methods.

The invention further relates to compositions, preferably immunogenic compositions, comprising an RSV F protein, a nucleic acid molecule and/or a vector, as described herein, and to the use thereof in inducing an immune response against RSV F protein, in particular to the use thereof as a vaccine against RSV. The invention also relates to methods for inducing an anti-respiratory syncytial virus (RSV) immune response in a subject, comprising

administering to the subject an effective amount of a pre-fusion RSV F protein, a nucleic acid molecule encoding said RSV F protein, and/or a vector comprising said nucleic acid molecule, as described herein. Preferably, the induced immune response is characterized by the induction of neutralizing antibodies to RSV and/or protective immunity against RSV. In a particular aspect, the invention relates to a method for inducing anti-respiratory syncytial virus (RSV) F antibodies in a subject, comprising administering to the subject an effective amount of an immunogenic composition comprising a pre-fusion RSV F protein, a nucleic acid molecule encoding said RSV F protein, and/or a vector comprising said nucleic acid molecule, as described herein.

## BRIEF DESCRIPTION OF THE FIGURES

FIG 1: shows a consensus amino acid sequence of the ectodomain of the RSV F protein of RSV subgroup A that was used for the construction of the subgroup A RSV F protein mutants described in the Examples. The precursor polypeptide contains a C-terminal extension with a linker and a foldon trimerization domain (underlined). The consensus RSV F sequence is very similar to the wild type (non-passaged) group A RSV F sequence (Kumaria *et al.* (2011).

FIG 2: shows a consensus amino acid sequence of the ectodomain of the RSV F protein of subgroup B that was used for the construction of the subgroup B RSV F protein mutants described in the Examples. The precursor polypeptide contains a C-terminal extension with a linker and a foldon trimerization domain (underlined). The consensus RSV F sequence is very similar to wild type (non-passaged) RSV F sequence (Kumaria *et al.* (2011).

FIG 3: Pre-fusion F stability of consensus F of subtype A and variants thereof. The content of F protein in the pre-fusion conformation was measured in the supernatant of transfected cells as described in Example 1. Supernatants of transfected cells were harvested, stored at 4 °C and tested for pre-fusion F content at 1, 4, 11 and 18 days of storage. The decay curves show that mutation I79M, P101S, P101Q, P101T, I152V or Q354L stabilize the pre-fusion F protein, as compared to the previously described stabilized subtype A F protein comprising the single D486N mutation (as described in WO2017/005844).

FIG 4: Pre-fusion F stability of consensus F of subtype B and variants thereof. The content of F protein in the pre-fusion conformation was measured in the supernatant of transfected cells as described in Example 1. Supernatants of transfected cells were harvested, stored at 4 degrees and tested for prefusion F content at 1, 4, 11 and 18 days of storage. The decay curves show that mutation T152M and K226M stabilize the pre-fusion F protein compared with the subtype B F-D486N (as described in WO2017/005844).

FIG.5: Purification of RSV pre-F (RSV180305; SEQ ID NO: 20). This variant contains the D486N substitution and two additional substitutions according to the invention that further stabilize the pre-fusion conformation (P101Q, and I152V) and purification of RSV pre-F (RSV172527; SEQ ID NO: 21) stabilized in the pre-fusion conformation by the mutations S46G, L203I, S215P, T357R, N371Y, D486N, and D489Y and which contained the additional mutations P101Q and I152V according to the invention. (A) and (B): RSV180305 contains a C-terminal C-tag and was purified from 300 ml Hek293F cells as described in Example 2. The arrows indicate the pooled fractions (see Fig. 5B). Purified RSV180305 (C) and RSV172527 (D) were analyzed by SEC-MALS after storage at 4°C for 4 weeks.

FIG.6: SDS-PAGE analysis of RSV172527 (SEQ ID NO: 21), protein sample containing pooled peak from the SEC chromatogram under non-reducing (lane 2) and reducing (lane 4) conditions. The gel is stained with Coomassie Brilliant Blue.

FIG. 7: ELISA of purified pre-F proteins RSV180305 (SEQ ID NO:20) and RSV172527 (SEQ ID NO: 21) compared to the control pre-fusion F protein RSV150042 (SEQ ID NO: 22) (e.g. PRPM as described in WO2017/174568) and were tested for binding to monoclonal antibodies CR9501 and CR9502 which are specific for the pre-fusion conformation of RSV F (which comprise the variable regions of the antibodies 58C5 and 30D8 as described in WO2012/006596, respectively), and Mab CR9506 that binds to both the pre- and post-fusion conformation of RSV F, and comprises a heavy chain variable region comprising SEQ ID NO: 25, and a light chain variable region comprising SEQ ID NO: 26. Plotted as Mean±SE.

FIG. 8: Temperature stability of purified pre-F proteins (A) RSV180305 and (B) RSV172527. Melting temperature (Tm °C) determined by differential scanning fluorimetry (DSF) assay with SyproOrange fluorescent dye (as described in Example 5).

**DETAILED DESCRIPTION OF THE INVENTION**

The fusion protein (F protein) of the respiratory syncytial virus (RSV) is involved in fusion of the viral membrane with a host cell membrane, which is required for infection. RSV F mRNA is translated into a 574 amino acid precursor protein designated F0, which contains a signal peptide sequence at the N-terminus (e.g. amino acid residues 1-26 of SEQ ID NO: 13 or 14) which is removed by a signal peptidase in the endoplasmic reticulum. F0 is cleaved at two sites (between amino acid residues 109/110 and 136/137) by cellular proteases (in

particular furin, or furin-like proteases) removing a short glycosylated intervening sequence (also referred to a p27 region, comprising the amino acid residues 110 to 136, and generating two domains (or subunits) designated F1 and F2. The F1 domain (amino acid residues 137-574) contains a hydrophobic fusion peptide at its N-terminus and the C-terminus contains the transmembrane (TM) (amino acid residues 530-550) and cytoplasmic region (amino acid residues 551-574). The F2 domain (amino acid residues 27-109) is covalently linked to F1 by two disulfide bridges. The F1-F2 heterodimers are assembled as homotrimers in the virion.

As described above, a vaccine against RSV infection is currently not yet available. One potential approach to producing a vaccine is providing a subunit vaccine based on purified RSV F protein. However, for this approach it is desirable that the purified RSV F protein is in a conformation which resembles the conformation of the pre-fusion state of RSV F protein and is stable over time, i.e. remains in the pre-fusion conformation, e.g. as determined by specific binding of the RSV F protein to antibodies that are specific for the pre-fusion conformation to the RSV F protein, and can be produced in sufficient quantities. In addition, for a soluble, subunit-based protein vaccine, the RSV F protein needs to be truncated by deletion of the transmembrane (TM) and the cytoplasmic region to create a soluble secreted F protein (sF protein). Because the TM region is responsible for membrane anchoring and increases stability, the anchorless soluble F protein is considerably more labile than the full-length protein and will even more readily refold into the post-fusion end-state. In order to obtain soluble F protein in the stable pre-fusion conformation that shows high expression levels and high stability, the pre-fusion conformation thus needs to be stabilized. Because also the full-length (membrane-bound) RSV F protein is metastable, stabilization of the pre-fusion conformation is also desirable for the full-length RSV F protein, i.e. including the TM and cytoplasmic region, e.g. for any live attenuated or vector-based vaccine approach.

For the stabilization of soluble RSV F, that is cleaved into the F1 and F2 subunit, in the pre-fusion conformation, a fibritin – based trimerization domain was fused to the C-terminus of the soluble RSV-F C-terminal end (McLellan et al., (2010, 2013)). This fibritin domain or 'Foldon' is derived from T4 fibritin and was described earlier as an artificial natural trimerization domain (Letarov *et al.*, (1993); S-Guthe *et al.*, (2004)). However, fusion of this trimerization domain alone does not result in stable pre-fusion RSV-F protein (Krarup *et al.*, (2015)). Indeed, these efforts have not yet resulted in candidates suitable for testing in humans.

Recently, we described combinations of several mutations that stabilize the RSV F protein in the pre-fusion conformation (e.g. WO2014/174018 and WO2014/202570). Thus, stable pre-fusion RSV F proteins have been described comprising e.g. a mutation of the amino acid residue on position 215, preferably a mutation of amino acid S on position 215 into P. In addition, soluble pre-fusion RSV F proteins have been described comprising a truncated F1 domain and comprising a mutation of the amino acid residue on position 215, preferably a mutation of amino acid residue S on position 215 into P, wherein the protein comprises a heterologous trimerization domain linked to said truncated F1 domain. Additional pre-fusion RSV F proteins have been described, wherein the proteins comprise one or more other stabilizing mutations, such as a mutation of the amino acid residue D on position 486 into N, a mutation of the amino acid residue L on position 203 into I, a mutation of the amino acid residue T on position 357 into K or R and/or a mutation of amino acid residue N at position 371 into Y, optionally in combination with the mutation on position 215. Further pre-fusion RSV F proteins have been described, wherein the proteins comprise yet one or more other mutations, such as a mutation of the amino acid residue D on position 489 into Y, a mutation of the amino acid residue S on position 398 into L and/or mutation of amino acid K on position 394 into R.

The present invention provides novel recombinant pre-fusion respiratory syncytial virus (RSV) Fusion (F) proteins comprising one or more stabilizing amino acids, wherein the one or more stabilizing amino acids are present on position 79, 101, 152, 226, and/or on position 354, optionally in combination with a stabilizing amino acid on position 486. According to the present invention it has been found that the presence of one or more specific stabilizing amino acids on positions 79, 101, 152, 354 and/or 226 (numbering according to SEQ ID NO 13 or 14), optionally in combination with the presence of the amino acid residue N on position 486, stabilizes the protein in the pre-fusion conformation.

According to the invention it has thus been demonstrated that the presence of the specific stabilizing amino acids on the indicated positions increases the stability of the proteins in the pre-fusion conformation. According to the invention, the specific amino acids can be either already present in the amino acid sequence or can be introduced by substitution (mutation) of the amino acid on that position into the specific amino acid according to the invention.

In certain embodiments, the present invention provides recombinant F proteins comprising an amino acid sequence of an RSV F protein, wherein the amino acid on position 79 is M, the amino acid on position 101 is S, Q or T, the amino acid on position 152 is V or M, the amino acid on position 226 is M, and/or the amino acid on position 354 is L, and wherein optionally the amino acid on position 486 is N.

In certain embodiments, the present invention provides recombinant F proteins of RSV subgroup A, comprising an amino acid sequence of an RSV F protein, wherein the amino acid on position 79 is M, the amino acid on position 101 is S, Q or T, the amino acid on position 152 is V or M, and/or the amino acid on position 354 is L, and wherein optionally the amino acid on position 486 is N.

In certain embodiments, the present invention provides recombinant F proteins of RSV subgroup B, comprising an amino acid sequence of an RSV F protein, wherein the amino acid on position 152 is V or M, and/or the amino acid on position 226 is M, and wherein optionally the amino acid on position 486 is N.

In certain embodiments, the present invention provides recombinant pre-fusion F proteins comprising one or more stabilizing mutations selected from the group consisting of: a mutation of the amino acid residue on position 79, a mutation of the amino acid residue on position 101, a mutation of the amino acid residue on position 152, a mutation of the amino acid residue on position 226 and a mutation of the amino acid residue on position 354, optionally in combination with a mutation of the amino acid on position 486.

In a specific embodiment, the present invention provides recombinant pre-fusion F proteins comprising a mutation of the amino acid residue on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N), in combination with one or more stabilizing mutations selected from the group consisting of: a mutation of the amino acid residues on position 79, a mutation of the amino acid residue on position 101, a mutation of the amino acid residue on position 152, a mutation of the amino acid residue on position 354 and a mutation of the amino acid residue on position 226.

In certain embodiments, the present invention provides recombinant pre-fusion F proteins of RSV subgroup A, comprising one or more stabilizing mutations selected from the group consisting of: a mutation of the amino acid residues on position 79, a mutation of the amino acid residue on position 101, a mutation of the amino acid residue on position 152 , and a mutation of the amino acid residue on position 354, optionally in combination with a mutation of the amino acid residue on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N).

In certain embodiments, the present invention provides recombinant pre-fusion F proteins of RSV subgroup B, comprising a mutation of the amino acid residue on position 152 and/or a mutation of the amino acid residue on position 226, optionally in combination with a mutation of the amino acid residue on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N).

In certain embodiments, the mutation of the amino acid residue on position 79 is a mutation of the amino acid isoleucine (I) into methionine (M) (I79M).

In certain embodiments, the mutation of the amino acid residue on position 101 is a mutation of the amino acid proline (P) into serine (S), glutamine (Q) or threonine (T) (P101S, P101Q, or P101T).

In certain embodiments, the mutation of the amino acid residue on position 152 is a mutation of the amino acid isoleucine (I) into valine (V) or methionine (M) (I152V or I152M).

In certain embodiments, the mutation of the amino acid residue on position 354 is a mutation of the amino acid glutamine (Q) into leucine (L) (Q354L).

In certain embodiments, the mutation of the amino acid residue on position 226 is a mutation of the amino acid lysine (K) into methionine (M) (K226M).

In certain embodiments, the pre-fusion F proteins comprise one or more additional stabilizing mutations selected from the group consisting of:

(a) a mutation of the amino acid residue S on position 46 into G;

(b) a mutation of amino acid residue L on position 203 into I;

(c) a mutation of amino acid residue S on position 215 into P;

(d) a mutation of the amino acid T on position 357 into K;

(e) a mutation of the amino acid N on position 371 into Y;

(f) a mutation of the amino acid residue E on position 487 into Q, N or I; and

(g) a mutation of the amino acid residue D on position 489 into Y; and

In certain embodiments, the recombinant pre-fusion F proteins comprise two or more stabilizing amino acids. In certain embodiments, the recombinant pre-fusion F proteins comprise two or more stabilizing mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise three or more stabilizing amino acids. In certain embodiments, the recombinant pre-fusion F proteins comprise three or more stabilizing mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise four or more stabilizing amino acids or mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise five or more stabilizing amino acids or mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise six or more stabilizing amino acids or mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise seven or more stabilizing amino acids or mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise eight or more stabilizing amino acids or mutations.

In certain embodiments, the recombinant pre-fusion F proteins comprise nine or more stabilizing amino acids or mutations.

In a preferred embodiment, the recombinant pre-fusion F protein is from RSV subgroup A and comprises at least a mutation of the amino acid P on position 101 into Q and a mutation of the amino acid I on position 152 into V, optionally in combination with a mutation of the amino acid residue S on position 46 into G, a mutation of the amino acid residue L on position 203 into I, a mutation of the amino acid residue S on position 215 into P, a mutation of the amino acid residue T on position 357 into R, a mutation of the amino

acid residue N on position 371 into Y, a mutation of the amino acid residue D on position 486 into N, and a mutation of the amino acid residue D on position 489 into Y.

In a preferred embodiment, the recombinant pre-fusion F protein is from RSV subgroup B and comprises at least a mutation of the amino acid I on position 152 into M and a mutation of the amino acid K on position 226 into M, optionally in combination with a mutation of the amino acid residue S on position 46 into G, a mutation of the amino acid residue L on position 203 into I, a mutation of the amino acid residue S on position 215 into P, a mutation of the amino acid residue T on position 357 into R, a mutation of the amino acid residue N on position 371 into Y, a mutation of the amino acid residue D on position 486 into N, and a mutation of the amino acid residue D on position 489 into Y.

The present invention thus provides new recombinant stable pre-fusion RSV F proteins, i.e. RSV F proteins that are stabilized in the pre-fusion conformation, or fragments thereof. The stable pre-fusion RSV F proteins of the invention, or fragments thereof, are in the pre-fusion conformation, i.e. they comprise (display) at least one epitope that is specific to the pre-fusion conformation F protein. An epitope that is specific to the pre-fusion conformation F protein is an epitope that is not present in the post-fusion conformation. Without wishing to be bound by any particular theory, it is believed that the pre-fusion conformation of RSV F protein may contain epitopes that are the same as those on the RSV F protein expressed on natural RSV virions, and therefore may provide advantages for eliciting protective neutralizing antibodies.

In certain embodiments, the pre-fusion RSV F proteins of the invention, or fragments thereof, comprise at least one epitope that is recognized by a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 1, a heavy chain CDR2 region of SEQ ID NO: 2, a heavy chain CDR3 region of SEQ ID NO: 3 and a light chain CDR1 region of SEQ ID NO: 4, a light chain CDR2 region of SEQ ID NO: 5, and a light

chain CDR3 region of SEQ ID NO: 6 (hereafter referred to as CR9501) and/or a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 7, a heavy chain CDR2 region of SEQ ID NO: 8, a heavy chain CDR3 region of SEQ ID NO: 9 and a light chain CDR1 region of SEQ ID NO: 10, a light chain CDR2 region of SEQ ID NO: 11, and a light chain CDR3 region of SEQ ID NO: 12 (referred to as CR9502) (Table 2). CR9501 and CR9502 comprise the heavy and light chain variable regions, and thus the binding specificities, of the antibodies 58C5 and 30D8, respectively, which have previously been shown to bind specifically to RSV F protein in its pre-fusion conformation and not to the post-fusion conformation (see WO2012/006596).

The present invention also provides methods for stabilizing the pre-fusion conformation of a RSV F protein, said methods comprising: introducing one or more stabilizing mutations into the RSV F protein, wherein the one or more stabilizing mutations are selected from the group consisting of:

a mutation of the amino acid residue on position 79, a mutation of the amino acid residue on position 101, a mutation of the amino acid residue on position 152, a mutation of the amino acid residue on position 354 and a mutation of the amino acid residue on position 226, optionally in combination with a mutation of the amino acid on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N).

In certain embodiments, the invention provides methods for stabilizing a RSV F protein of subgroup A, said methods comprising: introducing one or more stabilizing mutations selected from the group consisting of: a mutation of the amino acid residues on position 79, a mutation of the amino acid residue on position 101, and a mutation of the amino acid residue on position 354, optionally in combination with a mutation of the amino acid residue on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N).

In certain embodiments, the invention provides methods for stabilizing a RSV F protein of subgroup B, said methods comprising: introducing a mutation of the amino acid residue on position 152 and/or a mutation of the amino acid residue on position 226, optionally in combination with a mutation of the amino acid residue on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N).

In certain embodiments, the mutation of the amino acid residue on position 79 is a mutation of the amino acid isoleucine (I) into methionine (M) (I79M).

In certain embodiments, the mutation of the amino acid residue on position 101 is a mutation of the amino acid proline (P) into serine (S), glutamine (Q) or threonine (T) (P101S, P101Q, or P101T).

In certain embodiments, the mutation of the amino acid residue on position 152 is a mutation of the amino acid isoleucine (I) into valine (V) or methionine (M) (I152V or I152M).

In certain embodiments, the mutation of the amino acid residue on position 354 is a mutation of the amino acid glutamine (Q) into leucine (L) (Q354L).

In certain embodiments, the mutation of the amino acid residue on position 226 is a mutation of the amino acid lysine (K) into methionine (M) (K226M).

In certain embodiments, the methods comprise introducing one or more additional stabilizing mutations selected from the group consisting of:

(a) a mutation of the amino acid residue S on position 46 into G;

(b) a mutation of amino acid residue L on position 203 into I;

(c) a mutation of amino acid residue S on position 215 into P;

(d) a mutation of the amino acid T on position 357 into K;

(e) a mutation of the amino acid N on position 371 into Y;

(f) a mutation of the amino acid residue E on position 487 into Q, N or I; and

(g) a mutation of the amino acid residue D on position 489 into Y;

In certain embodiments, the methods comprise introducing two or more stabilizing mutations.

In certain embodiments, the methods comprise introducing three or more stabilizing mutations.

In certain embodiments, the methods comprise introducing four or more stabilizing mutations.

In certain embodiments, the methods comprise introducing five or more stabilizing mutations.

In certain embodiments, the methods comprise introducing six or more stabilizing mutations.

In certain embodiments, the methods comprise introducing seven or more stabilizing mutations.

In certain embodiments, the methods comprise introducing eight or more stabilizing mutations.

In certain embodiments, the methods comprise introducing nine or more stabilizing mutations.

In a preferred embodiment, the RSV F protein is from subgroup A and the method comprises introducing at least a mutation of the amino acid P on position 101 into Q and a mutation of the amino acid I on position 152 into V.

In another preferred embodiment, the RSV F protein is from subgroup B and the method comprises introducing at least a mutation of the amino acid T on position 152 into M and a mutation of the amino acid K on position 226 into M.

The invention further provides recombinant pre-fusion RSV F proteins obtainable by said methods, as well as uses thereof as described herein.

In certain embodiments, the recombinant pre-fusion RSV F proteins of the current invention are trimeric proteins.

As indicated above, fragments of the pre-fusion RSV F protein are also encompassed by the present invention. The fragment may result from either or both of amino-terminal (e.g. by cleaving off the signal sequence) and carboxy-terminal deletions (e.g. by deleting the transmembrane region and/or cytoplasmic tail). The fragment may be chosen to comprise an immunologically active fragment of the F protein, i.e. a part that will give rise to an immune response in a subject. This can be easily determined using in silico, in vitro and/or in vivo methods, all routine to the skilled person.

In certain embodiments, the encoded proteins or fragments thereof according to the invention comprise a signal sequence, also referred to as leader sequence or signal peptide, corresponding to amino acids 1-26 of SEQ ID NO: 13 or 14. Signal sequences typically are short (e.g. 5-30 amino acids long) amino acid sequences present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway, and are typically cleaved by signal peptidase to generate a free signal peptide and a mature protein.

In certain embodiments, the proteins or fragments thereof according to the invention do not comprise a signal sequence.

In certain embodiments, the (fragments of the) pre-fusion RSV F proteins are soluble proteins (i.e. not membrane-bound). In certain embodiments, the stable pre-fusion RSV F proteins or fragments thereof according to the invention comprise a truncated F1 domain and comprise a heterologous trimerization domain or an assembly domain for higher order assemblies of trimers, linked to said truncated F1 domain. According to the invention, it was shown that by linking a heterologous trimerization domain to the C-terminal amino acid residue of a truncated F1 domain, combined with one or more of the stabilizing mutation(s)

as described herein, soluble RSV F proteins are provided that show high expression and that bind to pre-fusion-specific antibodies, indicating that the proteins are in the pre-fusion conformation.

In certain embodiments, the heterologous trimerization domain comprises the amino acid sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 15).

As described above, in certain embodiments, the proteins of the invention or fragments thereof comprise a truncated F1 domain. As used herein a "truncated" F1 domain refers to a F1 domain that is not a full length F1 domain, i.e. wherein either N-terminally or C-terminally one or more amino acid residues have been deleted. According to the invention, at least the transmembrane domain and cytoplasmic tail have been deleted to permit expression as a soluble ectodomain.

In certain embodiments, the trimerization domain is linked to amino acid residue 513 of the RSV F1 domain. In certain embodiments, the trimerization domain thus comprises SEQ ID NO: 15 and is either directly linked to amino acid residue 513 of the RSV F1 domain, or through a linker.

In certain embodiments, the level of expression of the pre-fusion RSV F proteins of the invention is increased, as compared to a wild-type RSV F protein and/or to an RSV F protein comprising a mutation of the amino acid D on position 486 into N (D486N). In certain embodiments the pre-fusion content (defined as fraction of F protein that binds to the prefusion–specific CR9501 antibody) was significantly higher 5 to 10 days after harvest of the proteins, as compared to the wildtype F protein without said stabilizing substitutions.

The pre-fusion RSV F proteins according to the invention are stabilized in the pre-fusion conformation by the presence of one or more of the stabilizing amino acids (either already present or introduced by mutations), i.e. do not readily change into the post-fusion

conformation upon processing of the proteins, such as e.g. purification, freeze-thaw cycles, and/or storage etc.

In certain embodiments, the pre-fusion RSV F proteins according to the invention have an increased stability upon storage a 4°C as compared to a RSV F protein without the mutation(s). In certain embodiments, the proteins are stable upon storage at 4°C for at least 15, days, preferably at least 18 days, preferably at least 30 days, preferably at least 60 days, preferably at least 6 months, even more preferably at least 1 year. With "stable upon storage", it is meant that the proteins still display the at least one epitope specific for a pre-fusion specific antibody (e.g. CR9501 and/or CR9502) upon storage of the protein in solution (e.g. culture medium) at 4° C for at least 30 days. In certain embodiments, the proteins display the at least one pre-fusion specific epitope for at least 6 months, preferably for at least 1 year upon storage of the pre-fusion RSV F proteins at 4°C.

In certain embodiments, the pre-fusion RSV F proteins according to the invention have an increased thermostability as determined measuring the melting temperature as described in Example 6, as compared to RSV F proteins without said mutation(s).

In certain embodiments, the proteins display the at least one pre-fusion specific epitope after being subjected to 1 to 6 freeze-thaw cycles in an appropriate formulation buffer.

In certain embodiments, the proteins comprise a HIS-Tag, strep-tag or c-tag. A His-Tag or polyhistidine-tag is an amino acid motif in proteins that consists of at least five histidine (H) residues; a strep-tag is an amino acid sequence that consist of 8 residues (WSHPQFEK (SEQ ID NO: 23)); a c-tag is an amino acid motif that consists of 4 residues (EPEA; SEQ ID NO: 24). The tags are often at the N- or C-terminus of the protein and are generally used for purification purposes.

It is known that RSV exists as a single serotype having two antigenic subgroups: A and B. The amino acid sequences of the mature processed F proteins of the two groups are about 93% identical. As used throughout the present application, the amino acid positions are given in reference to a consensus sequence of the F protein of clinical isolates of subgroup A (SEQ ID NO: 13) or subgroup B (SEQ ID NO: 14). As used in the present invention, the wording "the amino acid at position "x" of the RSV F protein thus means the amino acid corresponding to the amino acid at position "x" in the RSV F protein of SEQ ID NO: 13 (for subgroup A) or SEQ ID NO: 14 (for subgroup B). Note that, in the numbering system used throughout this application 1 refers to the N-terminal amino acid of an immature F0 protein (SEQ ID NO: 13 or 14). When an F protein of another RSV strain is used, the amino acid positions of the F protein are to be numbered with reference to the numbering of the F protein of SEQ ID NO: 13 (for subgroup A) or SEQ ID NO: 14 (for subgroup B) by aligning the sequences of the other RSV strain with the F protein of SEQ ID NO: 13 or 14 with the insertion of gaps as needed. Sequence alignments can be done using methods well known in the art, e.g. by CLUSTALW, Bioedit or CLC Workbench.

As used throughout the present application nucleotide sequences are provided from 5' to 3' direction, and amino acid sequences from N-terminus to C-terminus, as custom in the art.

An amino acid according to the invention can be any of the twenty naturally occurring (or 'standard' amino acids). The standard amino acids can be divided into several groups based on their properties. Important factors are charge, hydrophilicity or hydrophobicity, size and functional groups. These properties are important for protein structure and protein–protein interactions. Some amino acids have special properties such as cysteine, that can form covalent disulfide bonds (or disulfide bridges) to other cysteine residues, proline that induces

turns of the protein backbone, and glycine that is more flexible than other amino acids. Table 1 shows the abbreviations and properties of the standard amino acids.

It will be appreciated by a skilled person that the mutations can be made to the protein by routine molecular biology procedures. The mutations according to the invention preferably result in increased expression levels and/or increased stabilization of the pre-fusion RSV F proteins as compared to RSV F proteins that do not comprise these mutation(s).

The present invention further provides nucleic acid molecules encoding the RSV F proteins according to the invention.

In preferred embodiments, the nucleic acid molecules encoding the proteins according to the invention are codon-optimized for expression in mammalian cells, preferably human cells. Methods of codon-optimization are known and have been described previously (e.g. WO 96/09378). A sequence is considered codon-optimized if at least one non-preferred codon as compared to a wild type sequence is replaced by a codon that is more preferred. Herein, a non-preferred codon is a codon that is used less frequently in an organism than another codon coding for the same amino acid, and a codon that is more preferred is a codon that is used more frequently in an organism than a non-preferred codon. The frequency of codon usage for a specific organism can be found in codon frequency tables, such as in http://www.kazusa.or.jp/codon. Preferably more than one non-preferred codon, preferably most or all non-preferred codons, are replaced by codons that are more preferred. Preferably the most frequently used codons in an organism are used in a codon-optimized sequence. Replacement by preferred codons generally leads to higher expression.

It will be understood by a skilled person that numerous different polynucleotides and nucleic acid molecules can encode the same protein as a result of the degeneracy of the genetic code. It is also understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the protein sequence encoded by the nucleic acid

molecules to reflect the codon usage of any particular host organism in which the proteins are to be expressed. Therefore, unless otherwise specified, a "nucleotide sequence encoding an amino acid sequence" includes all nucleotide sequences that are degenerate versions of each other and that encode the same amino acid sequence. Nucleotide sequences that encode proteins and RNA may or may not include introns.

Nucleic acid sequences can be cloned using routine molecular biology techniques, or generated de novo by DNA synthesis, which can be performed using routine procedures by service companies having business in the field of DNA synthesis and/or molecular cloning (e.g. GeneArt, GenScripts, Invitrogen, Eurofins).

The invention also provides vectors comprising a nucleic acid molecule as described above. In certain embodiments, a nucleic acid molecule according to the invention thus is part of a vector. Such vectors can easily be manipulated by methods well known to the person skilled in the art and can for instance be designed for being capable of replication in prokaryotic and/or eukaryotic cells. In addition, many vectors can be used for transformation of eukaryotic cells and will integrate in whole or in part into the genome of such cells, resulting in stable host cells comprising the desired nucleic acid in their genome. The vector used can be any vector that is suitable for cloning DNA and that can be used for transcription of a nucleic acid of interest. Suitable vectors according to the invention are e.g. adenovectors, alphavirus, paramyxovirus, vaccinia virus, herpes virus, retroviral vectors etc. The person skilled in the art is capable of choosing suitable expression vectors, and inserting the nucleic acid sequences of the invention in a functional manner.

Host cells comprising the nucleic acid molecules encoding the pre-fusion RSV F proteins form also part of the invention. The pre-fusion RSV F proteins may be produced through recombinant DNA technology involving expression of the molecules in host cells, e.g. Chinese hamster ovary (CHO) cells, tumor cell lines, BHK cells, human cell lines such as

HEK293 cells, PER.C6 cells, or yeast, fungi, insect cells, and the like, or transgenic animals or plants. In certain embodiments, the cells are from a multicellular organism, in certain embodiments they are of vertebrate or invertebrate origin. In certain embodiments, the cells are mammalian cells. In certain embodiments, the cells are human cells. In general, the production of a recombinant proteins, such the pre-fusion RSV F proteins of the invention, in a host cell comprises the introduction of a heterologous nucleic acid molecule encoding the protein in expressible format into the host cell, culturing the cells under conditions conducive to expression of the nucleic acid molecule and allowing expression of the protein in said cell. The nucleic acid molecule encoding a protein in expressible format may be in the form of an expression cassette, and usually requires sequences capable of bringing about expression of the nucleic acid, such as enhancer(s), promoter, polyadenylation signal, and the like. The person skilled in the art is aware that various promoters can be used to obtain expression of a gene in host cells. Promoters can be constitutive or regulated, and can be obtained from various sources, including viruses, prokaryotic, or eukaryotic sources, or artificially designed.

Cell culture media are available from various vendors, and a suitable medium can be routinely chosen for a host cell to express the protein of interest, here the pre-fusion RSV F proteins. The suitable medium may or may not contain serum.

A "heterologous nucleic acid molecule" (also referred to herein as 'transgene') is a nucleic acid molecule that is not naturally present in the host cell. It is introduced into for instance a vector by standard molecular biology techniques. A transgene is generally operably linked to expression control sequences. This can for instance be done by placing the nucleic acid encoding the transgene(s) under the control of a promoter. Further regulatory sequences may be added. Many promoters can be used for expression of a transgene(s), and are known to the skilled person, e.g. these may comprise viral, mammalian, synthetic promoters, and the like. A non-limiting example of a suitable promoter for obtaining

expression in eukaryotic cells is a CMV-promoter (US 5,385,839), e.g. the CMV immediate early promoter, for instance comprising nt. –735 to +95 from the CMV immediate early gene enhancer/promoter. A polyadenylation signal, for example the bovine growth hormone polyA signal (US 5,122,458), may be present behind the transgene(s). Alternatively, several widely used expression vectors are available in the art and from commercial sources, e.g. the pcDNA and pEF vector series of Invitrogen, pMSCV and pTK-Hyg from BD Sciences, pCMV-Script from Stratagene, etc, which can be used to recombinantly express the protein of interest, or to obtain suitable promoters and/or transcription terminator sequences, polyA sequences, and the like.

The cell culture can be any type of cell culture, including adherent cell culture, e.g. cells attached to the surface of a culture vessel or to microcarriers, as well as suspension culture. Most large-scale suspension cultures are operated as batch or fed-batch processes because they are the most straightforward to operate and scale up. Nowadays, continuous processes based on perfusion principles are becoming more common and are also suitable. Suitable culture media are also well known to the skilled person and can generally be obtained from commercial sources in large quantities, or custom-made according to standard protocols. Culturing can be done for instance in dishes, roller bottles or in bioreactors, using batch, fed-batch, continuous systems and the like. Suitable conditions for culturing cells are known (see e.g. Tissue Culture, Academic Press, Kruse and Paterson, editors (1973), and R.I. Freshney, Culture of animal cells: A manual of basic technique, fourth edition (Wiley-Liss Inc., 2000, ISBN 0-471-34889-9)).

The invention further provides compositions comprising a pre-fusion RSV F protein and/or a nucleic acid molecule, and/or a vector, as described above. The invention thus provides compositions comprising a pre-fusion RSV F protein that displays an epitope that is present in a pre-fusion conformation of the RSV F protein but is absent in the post-fusion

conformation. The invention also provides compositions comprising a nucleic acid molecule and/or a vector, encoding such pre-fusion RSV F protein. The invention further provides immunogenic compositions comprising a pre-fusion RSV F protein, and/or a nucleic acid molecule, and/or a vector, as described above. The invention further provides pharmaceutical compositions comprising a pre-fusion RSV F protein, and/or a nucleic acid molecule, and/or a vector, as described above and one or more pharmaceutically acceptable excipients.

The invention also provides the use of a stabilized pre-fusion RSV F protein, a nucleic acid molecule, and/or a vector, according to the invention, for inducing an immune response against RSV F protein in a subject. Further provided are methods for inducing an immune response against RSV F protein in a subject, comprising administering to the subject a pre-fusion RSV F protein, and/or a nucleic acid molecule, and/or a vector, according to the invention. Also provided are pre-fusion RSV F proteins, nucleic acid molecules, and/or vectors, according to the invention for use in inducing an immune response against RSV F protein in a subject. Further provided is the use of the pre-fusion RSV F proteins, and/or nucleic acid molecules, and/or vectors according to the invention for the manufacture of a medicament for use in inducing an immune response against RSV F protein in a subject.

The pre-fusion RSV F proteins, nucleic acid molecules, or vectors of the invention may be used for prevention (prophylaxis) and/or treatment of RSV infections. In certain embodiments, the prevention and/or treatment may be targeted at patient groups that are susceptible RSV infection. Such patient groups include, but are not limited to e.g., the elderly (e.g. $\geq$ 50 years old, $\geq$ 60 years old, and preferably $\geq$ 65 years old), the young (e.g. $\leq$ 5 years old, $\leq$ 1 year old), pregnant women (for maternal immunization), hospitalized patients and patients who have been treated with an antiviral compound but have shown an inadequate antiviral response.

The pre-fusion RSV F proteins, nucleic acid molecules and/or vectors according to the invention may be used e.g. in stand-alone treatment and/or prophylaxis of a disease or condition caused by RSV, or in combination with other prophylactic and/or therapeutic treatments, such as (existing or future) vaccines, antiviral agents and/or monoclonal antibodies.

The invention further provides methods for preventing and/or treating RSV infection in a subject utilizing the pre-fusion RSV F proteins, nucleic acid molecules and/or vectors according to the invention. In a specific embodiment, a method for preventing and/or treating RSV infection in a subject comprises administering to a subject in need thereof an effective amount of a pre-fusion RSV F protein, nucleic acid molecule and/or a vector, as described above. A therapeutically effective amount refers to an amount of a protein, nucleic acid molecule or vector, that is effective for preventing, ameliorating and/or treating a disease or condition resulting from infection by RSV. Prevention encompasses inhibiting or reducing the spread of RSV or inhibiting or reducing the onset, development or progression of one or more of the symptoms associated with infection by RSV. Amelioration as used in herein may refer to the reduction of visible or perceptible disease symptoms, viremia, or any other measurable manifestation of influenza infection.

For administering to subjects, such as humans, the invention may employ pharmaceutical compositions comprising a pre-fusion RSV F protein, a nucleic acid molecule and/or a vector as described herein, and a pharmaceutically acceptable carrier or excipient. In the present context, the term "pharmaceutically acceptable" means that the carrier or excipient, at the dosages and concentrations employed, will not cause any unwanted or harmful effects in the subjects to which they are administered. Such pharmaceutically acceptable carriers and excipients are well known in the art (see Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development

of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]). The RSV F proteins, or nucleic acid molecules, preferably are formulated and administered as a sterile solution although it may also be possible to utilize lyophilized preparations. Sterile solutions are prepared by sterile filtration or by other methods known per se in the art. The solutions are then lyophilized or filled into pharmaceutical dosage containers. The pH of the solution generally is in the range of pH 3.0 to 9.5, e.g. pH 5.0 to 7.5. The RSV F proteins typically are in a solution having a suitable pharmaceutically acceptable buffer, and the composition may also contain a salt. Optionally stabilizing agent may be present, such as albumin. In certain embodiments, detergent is added. In certain embodiments, the RSV F proteins may be formulated into an injectable preparation.

In certain embodiments, a composition according to the invention further comprises one or more adjuvants. Adjuvants are known in the art to further increase the immune response to an applied antigenic determinant. The terms “adjuvant” and "immune stimulant" are used interchangeably herein and are defined as one or more substances that cause stimulation of the immune system. In this context, an adjuvant is used to enhance an immune response to the RSV F proteins of the invention. Examples of suitable adjuvants include aluminium salts such as aluminium hydroxide and/or aluminium phosphate; oil-emulsion compositions (or oil-in-water compositions), including squalene-water emulsions, such as MF59 (see e.g. WO 90/14837); saponin formulations, such as for example QS21 and Immunostimulating Complexes (ISCOMS) (see e.g. US 5,057,540; WO 90/03184, WO 96/11711, WO 2004/004762, WO 2005/002620); bacterial or microbial derivatives, examples of which are monophosphoryl lipid A (MPL), 3-O-deacylated MPL (3dMPL), CpG-motif containing oligonucleotides, ADP-ribosylating bacterial toxins or mutants thereof, such as *E. coli* heat labile enterotoxin LT, cholera toxin CT, and the like; eukaryotic proteins (e.g.

antibodies or fragments thereof (e.g. directed against the antigen itself or CD1a, CD3, CD7, CD80) and ligands to receptors (e.g. CD40L, GMCSF, GCSF, etc), which stimulate immune response upon interaction with recipient cells. In certain embodiments the compositions of the invention comprise aluminium as an adjuvant, e.g. in the form of aluminium hydroxide, aluminium phosphate, aluminium potassium phosphate, or combinations thereof, in concentrations of 0.05 – 5 mg, e.g. from 0.075-1.0 mg, of aluminium content per dose.

The pre-fusion RSV F proteins may also be administered in combination with or conjugated to nanoparticles, such as e.g. polymers, liposomes, virosomes, virus-like particles. The pre-fusion F proteins may be combined with, encapsidated in or conjugated to the nanoparticles with or without adjuvant. Encapsulation within liposomes is described, e.g. in US 4,235,877. Conjugation to macromolecules is disclosed, for example in US 4,372,945 or US 4,474,757. The pre-fusion RSV F proteins may also be conjugated to self-assembling proteins.

In other embodiments, the compositions do not comprise adjuvants.

In certain embodiments, the invention provides methods for making a vaccine against respiratory syncytial virus (RSV), comprising providing an RSV F protein, nucleic acid or vector according to the invention and formulating it into a pharmaceutically acceptable composition. The term "vaccine" refers to an agent or composition containing an active component effective to induce a certain degree of immunity in a subject against a certain pathogen or disease, which will result in at least a decrease (up to complete absence) of the severity, duration or other manifestation of symptoms associated with infection by the pathogen or the disease. In the present invention, the vaccine comprises an effective amount of a pre-fusion RSV F protein and/or a nucleic acid molecule encoding a pre-fusion RSV F protein, and/or a vector comprising said nucleic acid molecule, which results in an immune response against the F protein of RSV. This provides a method of preventing serious lower

respiratory tract disease leading to hospitalization and the decrease in frequency of complications such as pneumonia and bronchiolitis due to RSV infection and replication in a subject. The term "vaccine" according to the invention implies that it is a pharmaceutical composition, and thus typically includes a pharmaceutically acceptable diluent, carrier or excipient. It may or may not comprise further active ingredients. In certain embodiments it may be a combination vaccine that further comprises other components that induce an immune response, e.g. against other proteins of RSV and/or against other infectious agents. The administration of further active components may for instance be done by separate administration or by administering combination products of the vaccines of the invention and the further active components.

Compositions may be administered to a subject, e.g. a human subject. The total dose of the RSV F proteins in a composition for a single administration can for instance be about 0.01 μg to about 10 mg, e.g. 1 μg – 1 mg, e.g. 10 μg – 100 μg. The total dose of the (adeno)vectors comprising DNA encoding the RSV F proteins in a composition for a single administration can for instance be about $0.1 \times 10^{10}$ vp/ml and $2 \times 10^{11}$, preferably between about $1 \times 10^{10}$ vp/ml and $2 \times 10^{11}$ vp/ml, preferably between $5 \times 10^{10}$ vp/ml and $1 \times 10^{11}$ vp/ml.

Administration of the compositions according to the invention can be performed using standard routes of administration. Non-limiting embodiments include parenteral administration, such as intradermal, intramuscular, subcutaneous, transcutaneous, or mucosal administration, e.g. intranasal, oral, and the like. In one embodiment a composition is administered by intramuscular injection. The skilled person knows the various possibilities to administer a composition, e.g. a vaccine in order to induce an immune response to the antigen(s) in the vaccine.

A subject as used herein preferably is a mammal, for instance a rodent, e.g. a mouse, a cotton rat, or a non-human-primate, or a human. Preferably, the subject is a human subject.

The proteins, nucleic acid molecules, vectors, and/or compositions may also be administered, either as prime, or as boost, in a homologous or heterologous prime-boost regimen. If a boosting vaccination is performed, typically, such a boosting vaccination will be administered to the same subject at a time between one week and one year, preferably between two weeks and four months, after administering the composition to the subject for the first time (which is in such cases referred to as 'priming vaccination'). In certain embodiments, the administration comprises a prime and at least one booster administration.

In addition, the proteins of the invention may be used as diagnostic tool, for example to test the immune status of an individual by establishing whether there are antibodies in the serum of such individual capable of binding to the protein of the invention. The invention thus also relates to an in vitro diagnostic method for detecting the presence of an RSV infection in a patient said method comprising the steps of a) contacting a biological sample obtained from said patient with a protein according to the invention; and b) detecting the presence of antibody-protein complexes.

**EXAMPLES**

EXAMPLE 1: *Stability of pre-fusion conformation of RSV F variants in culture supernatant*

The RSV F sequence that was used as a control for the stability study was based on either a consensus sequence for subgroup A (SEQ ID NO: 13) or a consensus sequence of subgroup B (SEQ ID NO: 14) since the consensus sequence will be very similar to wild-type (non-passaged) sequences corresponding to clinical isolates (Kumaria *et al.* ( 2011)). For detection purposes, the F proteins were C-terminally fused to a strep-tag. To assess the stability of various point mutations in RSV F, the content of F protein in the pre-fusion conformation was measured in AlphaLISA.

The assay was performed by adding 5 µl of RSV F sample to 25 µl of a mixture of pre-fusion – specific Mab CR9501 (0.8 nM), anti-human-IgG Acceptor bead and Streptactin Donor beads (Perkin Elmer) in assay buffer. Chemiluminescent emission at 615 nm was measured following 2.5 hours incubation at room temperature of the mixture. Only RSV F in the pre-fusion conformation is bound by both antibodies simultaneously and thus will give a signal in this assay. The measurements were done at days 0, 4, 11 and 18 after harvest and the decrease of the pre-fusion F signal was compared with the wt F signal and the signal of the F protein with the stabilizing D486N mutation. Unstable pre-fusion F protein can be identified by a time-dependent loss of CR9501 binding (F wt and F-D486N), while more stable pre-fusion constructs displayed either a higher pre-fusion F content at the day of harvest, and/or a slower or no decrease of pre-fusion F content after storage for a period at 4°C as compared to the control F protein (e.g. wtF and F-D486N) without the stabilizing point mutation of the present invention. Figure 3 shows that addition of one or more of the mutations V76G, I79M, P101S, P101Q, P101T, I152V or Q354L stabilizes the pre-fusion F protein compared with the subtype A F-D486N variant. Figure 4 shows that addition of mutation T152M and/or K226M stabilizes the pre-fusion F protein compared with the subtype B F-D486N variant.

EXAMPLE 2: *Preparation of stable pre-fusion RSV F polypeptides – stabilizing mutations*

A soluble pre-fusion protein based on the consensus RSV F (SEQ ID NO: 13 or 14) could not be purified due to the apparent instability (as shown in Figures 3, 4). To evaluate some of the stabilizing mutations shown in Figure 3, RSV-F protein (RSV180305; SEQ ID NO: 20) was produced with two mutations that stabilize the prefusion conformation, in particular the mutations P101Q and I152V, in addition to the previously described D486N stabilizing mutation (Krarup et. al., 2015). Additionally, another stabilized prefusion F protein was produced (RSV172527; SEQ ID NO: 21) in which the P101Q and I152V

substitutions were introduced in a pre-fusion RSV F protein that already contained several previously described stabilizing substitutions, in particular the mutations S46G, L203I, S215P, T357R, N371Y, D486N and D489Y. The constructs were synthesized and codon-optimized at Gene Art (Life Technologies, Carlsbad, CA). The constructs were cloned into pCDNA2004 or generated by standard methods widely known within the field involving site-directed mutagenesis and PCR and sequenced. The expression platform used was the HEK293 cells. The cells were transiently transfected using 293Fectin (Life Technologies) according to the manufacturer's instructions and cultured for 5 or 6 days at 37°C and 10% $CO_2$. The culture supernatant was harvested and spun for 5 minutes at 300 g to remove cells and cellular debris. The spun supernatant was subsequently sterile filtered using a 0.22 um vacuum filter and stored at 4°C until use.

Pre-fusion RSV F proteins were purified using a two-step purification protocol including either CaptureSelect™ C-tag affinity column for C-tagged protein RSV180305 or, for the non-tagged protein RSV172527, by cation-exchange at pH 5.0 (HiTrap Capto SP ImpRes column; GE Healthcare Biosciences, Pittsburgh, PA, USA) followed by anion exchange using Resource-Q column. Both RSV180305 (SEQ ID NO:20) and RSV172527 (SEQ ID NO: 21) were further purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare), as shown in Figure 5a and b respectively. After storage at 4°C for 4 weeks, SEC-MALS analysis of the pooled samples showed that the purified proteins were trimeric and it was also observed that the purified RSV180305 contained a minor fraction of aggregates.

EXAMPLE 3: *SDS–PAGE analysis.*

Purified RSV172527 was analyzed on 4–12% (w/v) Bis–Tris NuPAGE gels, 1 x MOPS (Life Technologies) under reducing or non-reducing conditions. All procedures were performed according to manufacturer's instructions. For purity analysis the gels were stained

with Krypton Infrared Protein Stain (Thermo Scientific). Non-reduced and reduced RSV172527 was pure and bands are visible at the expected height of the $F_0$ and $F_1$ ectodomain respectively.

EXAMPLE 4: *ELISA*

After storage at 4°C for 4 weeks, binding of the purified pre-fusion RSV F proteins RSV180305, RSV172725 and a control pre-fusion F protein (RSV150042) (SEQ ID NO: 22, as described previously in WO2017/174568, to pre-fusion-specific neutralizing antibodies was tested in ELISA. The 1/2 AreaPlate-96 HB plates (white, high protein binding affinity (PerkinElmer)) were coated with the test panel of anti RSV monoclonal antibodies. Mab CR9501 and CR9502 are specific for the pre-fusion conformation of RSV F and Mab CR9506 binds both the pre- and post-fusion conformation of RSV F. CR9506 competes with motavizumab (data not shown) and binds to the same linear epitope. The antibodies were diluted in PBS at 1 μg/ml and coated to the plates overnight at 4°C in PBS. The next day, plates were washed with washing buffer (PBS, 0.05% Tween) and blocked in PBS with 1% bovine serum albumin. All incubations were performed at room temperature for 1 h. After each step, plates were washed three times with the Wash buffer. Titrations of the purified RSV F protein were prepared in the washing buffer with 1% bovine serum albumin. CR9506 was biotinylated according to standard procedure and used at 0.05 μg/ml with BM Chemiluminescence ELISA substrate (POD) (Sigma Aldrich) for detection. As shown in Figure 7, all purified RSV F proteins bound in a similar fashion to the pre-fusion-specific Mabs (CR9501 and CR9502) and the Mab that is specific for both conformations (CR9506), thus showing that the F proteins according to the invention were in the pre-fusion conformation.

EXAMPLE 5: *Temperature stability of the RSV F proteins*

The temperature stability of the purified proteins was determined by differential scanning fluorometry (DSF). The purified pre-fusion F protein was mixed with SYPRO orange fluorescent dye (Life Technologies S6650) in a 96-well optical qPCR plate. The optimal dye and protein concentration was determined experimentally (data not shown). Protein dilutions were performed in PBS, and a negative control sample containing the dye only was used as a reference subtraction. The measurement was performed in a qPCR instrument (Applied Biosystems ViiA 7) using the following parameters: a temperature ramp from 25–95 °C with a rate of 0.015 °C per second. Data was collected continuously. The melting curves were plotted using GraphPad PRISM software (version 5.04). Melting temperatures were calculated at the 50% maximum of fluorescence using a non-linear EC50 shift equation. The melting temperature of RSV180305 was 65.9 degrees (Fig. 8a) and for RSV172527 was 72.9 (Fig 8b). The reference pre-fusion RSV F (RSV150042) has a melting temperature of 65.0 (data not shown).

**Table 1.** Standard amino acids, abbreviations and properties

| Amino Acid | 3-Letter | 1-Letter | Side chain polarity | Side chain charge (pH 7.4) |
|---|---|---|---|---|
| alanine | Ala | A | non-polar | Neutral |
| arginine | Arg | R | polar | Positive |
| asparagine | Asn | N | polar | Neutral |
| aspartic acid | Asp | D | polar | Negative |
| cysteine | Cys | C | non-polar | Neutral |
| glutamic acid | Glu | E | polar | Negative |
| glutamine | Gln | Q | polar | Neutral |
| glycine | Gly | G | non-polar | Neutral |
| histidine | His | H | polar | Positive (10%) neutral(90%) |
| isoleucine | Ile | I | non-polar | Neutral |
| leucine | Leu | L | non-polar | Neutral |
| lysine | Lys | K | polar | Positive |
| methionine | Met | M | non-polar | Neutral |
| phenylalanine | Phe | F | non-polar | Neutral |
| proline | Pro | P | non-polar | Neutral |
| serine | Ser | S | polar | Neutral |
| threonine | Thr | T | polar | Neutral |
| tryptophan | Trp | W | non-polar | Neutral |
| tyrosine | Tyr | Y | polar | Neutral |
| valine | Val | V | non-polar | Neutral |

**Table 2.** Amino acid sequences of antibodies CR9501 and CR9502

| Ab | VH domain | VH CDR1 | VH CDR2 | VH CDR3 |
|---|---|---|---|---|
| **CR9501** | Amino acids 1-125 of SEQ ID NO: 16 | GASINSDNYYWT (SEQ ID NO:1) | HISYTGNTYYTPSLKS (SEQ ID NO:2) | CGAYVLISNCGWFDS (SEQ ID NO:3) |
| **CR9502** | Amino acids 1-121 of SEQ ID NO:18 | GFTFSGHTIA (SEQ ID NO:7) | WVSTNNGNTEYAQKIQG (SEQ ID NO:8) | EWLVMGGFAFDH (SEQ ID NO:9) |

| Ab | VL domain | VL CDR1 | VL CDR2 | VL CDR3 |
|---|---|---|---|---|
| **CR9501** | Amino acids 1-107 of SEQ ID NO: 17 | QASQDISTYLN (SEQ ID NO: 4) | GASNLET (SEQ ID NO:5) | QQYQYLPYT (SEQ ID NO:6) |
| **CR9502** | Amino acids 1-110 of SEQ ID NO: 19 | GANNIGSQNVH (SEQ ID NO:10) | DDRDRPS (SEQ ID NO:11) | QVWDSSRDQAVI (SEQ ID NO:12) |

5

**Sequences**

RSV F A consensus full length (SEQ ID NO: 13)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITI
ELSNIKENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPAANNRARRELPRFM
NYTLNNAKKTNVTLSKKRKRRFLGFLLGVGSAIASGIAVSKVLHLEGEVNKIKSALL
STNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSISNIETVIEFQQKNNRLL
EITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIM
SIIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNA
GSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSS
SVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNK
QEGKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNVGK
STTNIMITTIIIVIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

RSV F B consensus ectodomain (SEQ ID NO: 14)

MELLIHRSSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNRARREAPQYM
NYTINTTKNLNVSISKKRKRRFLGFLLGVGSAIASGIAVSKVLHLEGEVNKIKNALLS
TNKAVVSLSNGVSVLTSKVLDLKNYINNQLLPIVNQQSCRISNIETVIEFQQKNSRLLE
ITREFSVNAGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSI
IKEEVLAYVVQLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSV
SFFPQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVIT
SLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEG
KNLYVKGEPIINYYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELL

**SEQ ID NO: 15 (fibritin)**

GYIPEAPRDGQAYVRKDGEWVLLSTFL

**CR9501 heavy chain (SEQ ID NO: 16):**

QVQLVQSGPGLVKPSQTLALTCNVSGASINSDNYYWTWIRQRPGGGLEWIGHISYTG
NTYYTPSLKSRLSMSLETSQSQFSLRLTSVTAADSAVYFCAACGAYVLISNCGWFDS
WGQGTQVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGAL
TSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

**CR9501 light chain (SEQ ID NO: 17):**

EIVMTQSPSSLSASIGDRVTITCQASQDISTYLNWYQQKPGQAPRLLIYGASNLETGVP
SRFTGSGYGTDFSVTISSLQPEDIATYYCQQYQYLPYTFAPGTKVEIKRTVAAPSVFIF
PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

**CR9502 heavy chain (SEQ ID NO: 18):**

EVQLLQSGAELKKPGASVKISCKTSGFTFSGHTIAWVRQAPGQGLEWMGWVSTNNG
NTEYAQKIQGRVTMTMDTSTSTVYMELRSLTSDDTAVYFCAREWLVMGGFAFDHW
GQGTLLTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC

**CR9502 light chain (SEQ ID NO: 19):**

QSVLTQASSVSVAPGQTARITCGANNIGSQNVHWYQQKPGQAPVLVVYDDRDRPSG
IPDRFSGSNSGNTATLTISRVEAGDEADYYCQVWDSSRDQAVIFGGGTKLTVLGQPK
AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQS
NNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTIAPTECS

**RSV180305 (SEQ ID NO:20)**

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIK
ENKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTQAANNRARRELPRFMNYTLNNAKKT
NVTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSV
LTSKVLDLKNYIDKQLLPIVNKQSCSISNIETVIEFQQKNNRLLEITREFSVNAGVTTPVSTYML
TNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWK
LHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSE
VNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYV
SNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPSNEFDASISQVNEKINQSLAFI
RKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFLGGSEPEA

**RSV172527 (SEQ ID NO: 21)**

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLGALRTGWYTSVITIELSNIK
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NVTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSV
LTSKVLDLKNYIDKQILPIVNKQSCSIPNIETVIEFQQKNNRLLEITREFSVNAGVTTPVSTYML
TNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWK
LHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAERCKVQSNRVFCDTMYSLTLPS
EVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDY
VSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPSNEFYASISQVNEKINQSLA
FIRKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFL

**RSV150042 (SEQ ID NO: 22)**

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIELSNIK
EIKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNYTLNNAKKTN
VTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVL
TSKVLDLKNYIDKQLLPIVNKQSCSIPNIETVIEFQQKNNRLLEITREFSVNAGVTTPVSTYMLT
NSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWKL
HTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSE
VNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYV
SNKGVDTVSVGNTLYYVNKQEGKSLYVKGEPIINFYDPLVFPSNEFDASISQVNEKINQSLAFI
RKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFL

**CR9506 heavy chain (SEQ ID NO: 25)**

EVQLVQSGAEVKKPGSSVKVSCKASGGTFSRSLITWVRQAPGQGLEWMGEISLVFGSAKNA
QKFQGRVTITADESTSTAHMEMISLKHEDTAVYYCAAHQYGSGTHNNFWDESELRFDLWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPA
VLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTHTCPPCPAPELL
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY
NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEM
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG
NVFSCSVMHEALHNHYTQKSLSLSPGK

**CR9506 light chain (SEQ ID NO: 26)**

DIVMTQSPSSLSASVGDRVTIACRASQSIGTYLNWYQQKPGKAPKLLIYAASSLQSGVPSRFS
GSGSGTHFTLAISSLQAEDFATYSCQQSYTIPYTFGQGTKLEIKRTVAAPSVFIFPPSDEQLKSG
TASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKH
KVYACEVTHQGLSSPVTKSFNRGEC

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Swanson, et al. (2011)

## Claims

1. A recombinant pre-fusion respiratory syncytial virus (RSV) Fusion (F) protein comprising one or more stabilizing amino acids, wherein the one or more stabilizing amino acids are present on position 79, 101, 152, 226, and/or on position 354, optionally in combination with a stabilizing amino acid on position 486.

2. The recombinant pre-fusion respiratory syncytial virus (RSV) Fusion (F) protein of claim 1, wherein the amino acid on position 79 is M, the amino acid on position 101 is S, Q or T, the amino acid on position 152 is V or M, the amino acid on position 226 is M, and/or the amino acid on position 354 is L, and wherein optionally the amino acid on position 486 is N.

3. The recombinant pre-fusion respiratory syncytial virus (RSV) Fusion (F) protein of claim 1 or 2, comprising one or more stabilizing mutations selected from the group consisting of a mutation of the amino acid residues on position 79, a mutation of the amino acid residue on position 101, a mutation of the amino acid residue on position 152, a mutation of the amino acid residue on position 354 and a mutation of the amino acid residue on position 226, optionally in combination with a mutation of the amino acid on position 486.

4. The recombinant pre-fusion F protein of claim 3, comprising a mutation of the amino acid residue on position 486, preferably a mutation of the amino acid D on position 486 into N (D486N), in combination with one or more stabilizing mutations selected from the group consisting of a mutation of the amino acid residues on position 79, a

mutation of the amino acid residue on position 101, a mutation of the amino acid residue on position 152, a mutation of the amino acid residue on position 354 and a mutation of the amino acid residue on position 226.

5. The recombinant pre-fusion F protein of claim 3 or 4, wherein the mutation of the amino acid residue on position 79 is a mutation of the amino acid isoleucine (I) into methionine (M).

6. The recombinant pre-fusion F protein of claim 3 or 4, wherein the mutation of the amino acid residue on position 101 is a mutation of the amino acid proline (P) into serine (S), glutamine (Q) or threonine (T).

7. The recombinant pre-fusion F protein of claim 3 or 4, wherein the mutation of the amino acid residue on position 152 is a mutation of the amino acid isoleucine (I) into valine (V) or methionine (M).

8. The recombinant pre-fusion F protein of claim 3 or 4, wherein the mutation of the amino acid residue on position 354 is a mutation of the amino acid glutamine (Q) into leucine (L).

9. The recombinant pre-fusion F protein of claim 3 or 4, wherein the mutation of the amino acid residue on position 226 is a mutation of the amino acid lysine (K) into methionine (M).

10. The recombinant pre-fusion F protein of any one of the claims 1-9, wherein the recombinant pre-fusion F protein comprises two or more of the stabilizing mutations.

11. The recombinant pre-fusion F protein of any one of the claims 1-9, wherein the recombinant pre-fusion F protein comprises three or more of the stabilizing mutations.

12. The recombinant pre-fusion F protein of any one of the claims 1-9, wherein the recombinant pre-fusion F protein comprises three or more of the stabilizing mutations.

13. The recombinant pre-fusion F protein of any one of the claims 1-9, wherein the recombinant pre-fusion F protein comprises four or more of the stabilizing mutations.

14. The recombinant pre-fusion F protein of any one of the claims 1-9, wherein the recombinant pre-fusion F protein comprises five or more of the stabilizing mutations.

15. The recombinant pre-fusion F protein of any one of the claims 1-9, wherein the recombinant pre-fusion F protein comprises six or more of the stabilizing mutations.

16. The pre-fusion RSV F protein according to any one of the preceding claims, wherein the protein comprises at least one epitope that is specific to the pre-fusion conformation F protein, wherein the at least one epitope is recognized by a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 1, a heavy chain CDR2 region of SEQ ID NO: 2, a heavy chain CDR3 region of SEQ ID NO: 3 and a light chain CDR1 region of SEQ ID NO: 4, a light chain CDR2 region of SEQ ID NO: 5, and a light chain CDR3 region of SEQ ID NO: 6 and/or a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO:

7, a heavy chain CDR2 region of SEQ ID NO: 8, a heavy chain CDR3 region of SEQ ID NO: 9 and a light chain CDR1 region of SEQ ID NO: 10, a light chain CDR2 region of SEQ ID NO: 67, and a light chain CDR3 region of SEQ ID NO: 11.

17. Pre-fusion RSV F protein according to any one of the preceding claims, wherein the protein is trimeric.

18. Pre-fusion RSV F protein according to any one of the preceding claims, comprising a truncated F1 domain and a heterologous trimerization domain linked to said truncated F1 domain.

19. Pre-fusion RSV F protein according to claim 18, wherein the heterologous trimerization domain comprises the amino acid sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 15).

20. Pre-fusion RSV F protein according to claim 18 or 19, wherein the trimerization domain is linked to amino acid residue 513 of the RSV F protein.

21. Nucleic acid molecule encoding a pre-fusion RSV F protein according to any one of the preceding claims 1-20.

22. Nucleic acid molecule according to claim 21, wherein the nucleic acid molecule has been codon-optimized for expression in mammalian cells.

23. Vector comprising a nucleic acid molecule according to claim 21 or claim 22.

24. Composition comprising a pre-fusion RSV F protein according to any of the claims 1-20, a nucleic acid molecule according to claim 21 or claim 22 and/or a vector according to claim 23.

25. Pre-fusion RSV F protein according to any of the claims 1-20, a nucleic acid molecule according to claim 21 or claim 22 and/or a vector according to claim 23 for use in inducing an immune response against RSV F protein.

26. Pre-fusion RSV F protein according to any of the claims 1-20, a nucleic acid molecule according to claim 21 or claim 22 and/or a vector according to claim 23 for use as a vaccine.

27. Pre-fusion RSV F protein according to any of the claims 1-20, a nucleic acid molecule according to claim 21 or claim 22 and/or a vector according to claim 23 for use in the prophylaxis and/or treatment of RSV infection.

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKG
YLSALRTGWYTSVITIELSNIKENKCNGTDAKVKLIKQELDKY
KNAVTELQLLMQSTPAANNRARRELPRFMNYTLNNAKKTNVTL
SKKRKRRFLGFLLGVGSAIASGIAVSKVLHLEGEVNKIKSALL
STNKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNKQSCSIS
NIETVIEFQQKNNRLLEITREFSVNAGVTTPVSTYMLTNSELL
SLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVV
QLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCD
NAGSVSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIF
NPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRG
IIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKGEP
IINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAI
GGYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO:
13)

Fig. 1

MELLIHRSSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRG
YFSALRTGWYTSVITIELSNIKETKCNGTDTKVKLIKQELDKY
KNAVTELQLLMQNTPAANNRARREAPQYMNYTINTTKNLNVSI
SKKRKRRFLGFLLGVGSAIASGIAVSKVLHLEGEVNKIKNALL
STNKAVVSLSNGVSVLTSKVLDLKNYINNQLLPIVNQQSCRIS
NIETVIEFQQKNSRLLEITREFSVNAGVTTPLSTYMLTNSELL
SLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVV
QLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRTDRGWYCD
NAGSVSFFPQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIF
NSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRG
IIKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEGKNLYVKGEP
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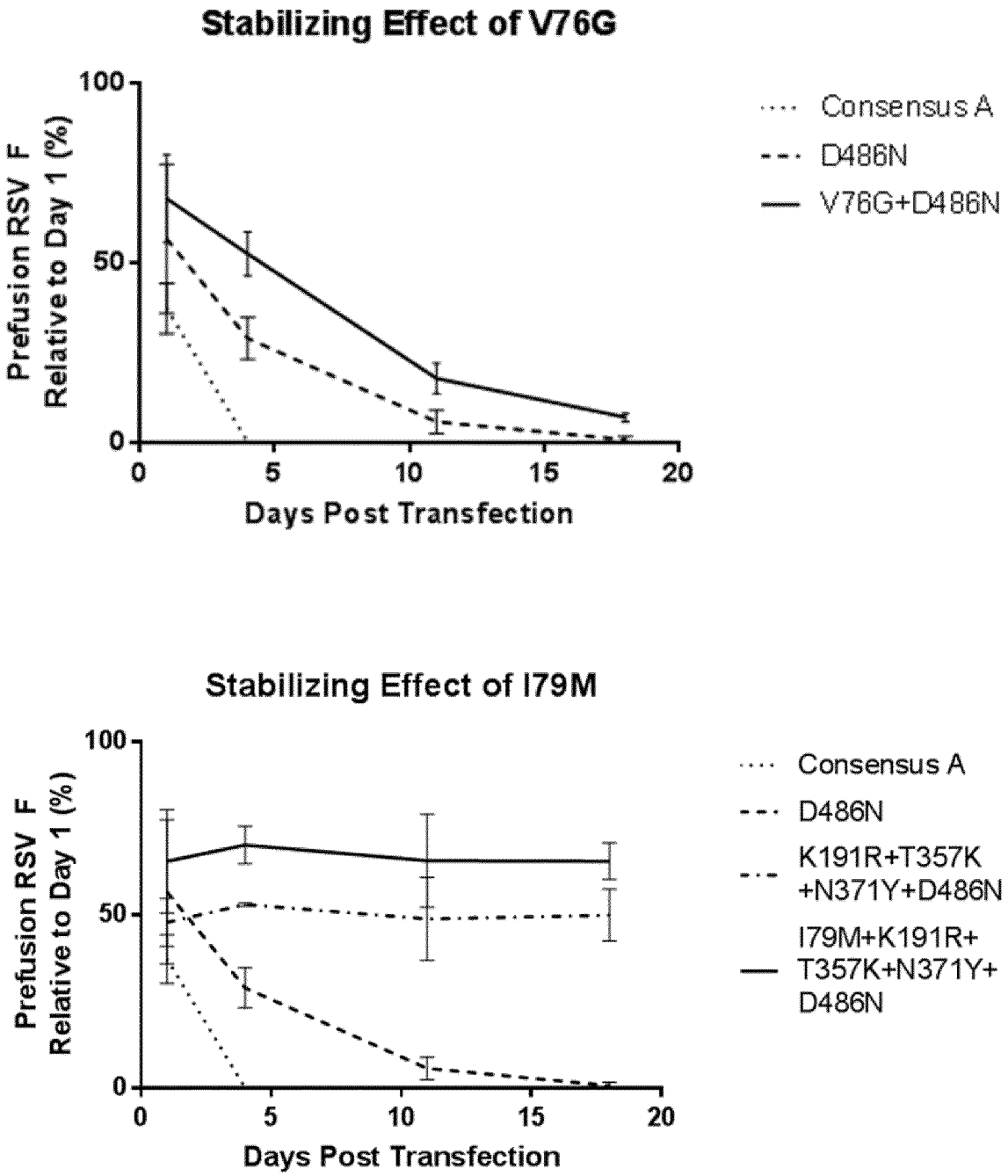
14)

Fig. 2


Stabilizing Effect of V76G
Prefusion RSV F
Relative to Day 1 (%)
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50
0
0
5
10
15
20
Days Post Transfection
Consensus A
D486N
V76G+D486N
Stabilizing Effect of I79M
Prefusion RSV F
Relative to Day 1 (%)
100
50
0
0
5
10
15
20
Days Post Transfection
Consensus A
D486N
K191R+T357K
+N371Y+D486N
I79M+K191R+
T357K+N371Y+
D486N

Fig. 3

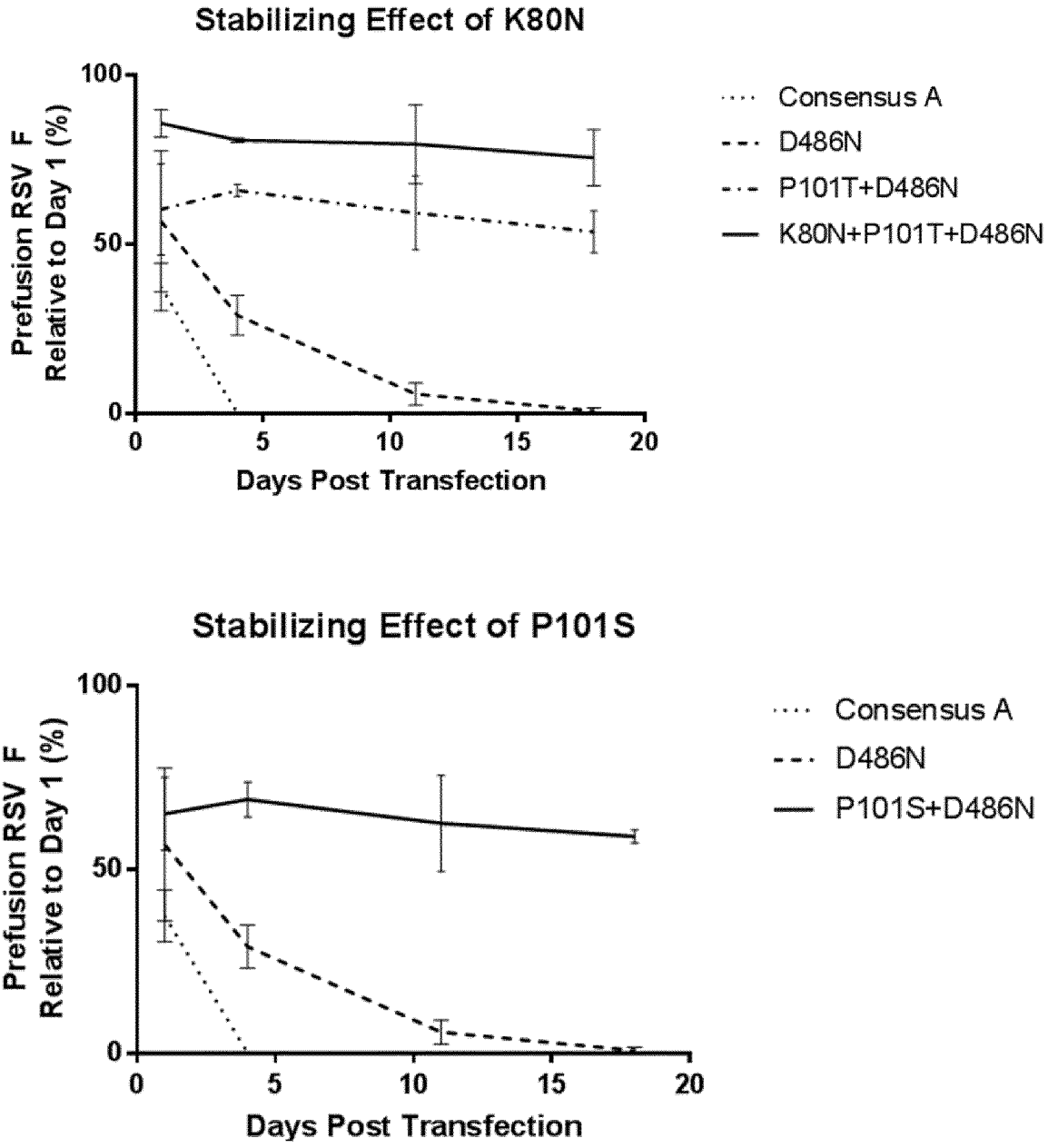



Fig. 3 - continued

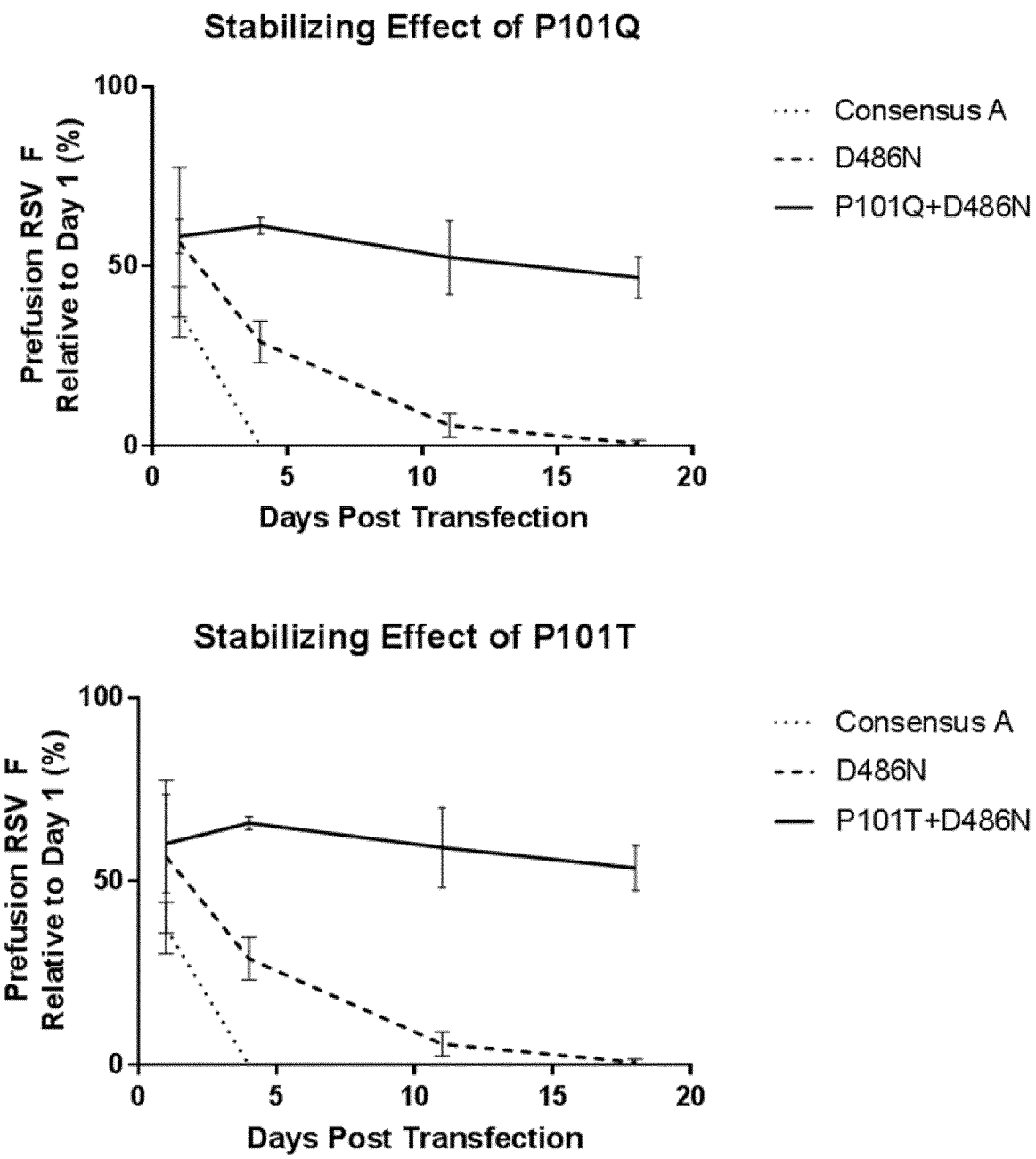



Fig. 3 - continued

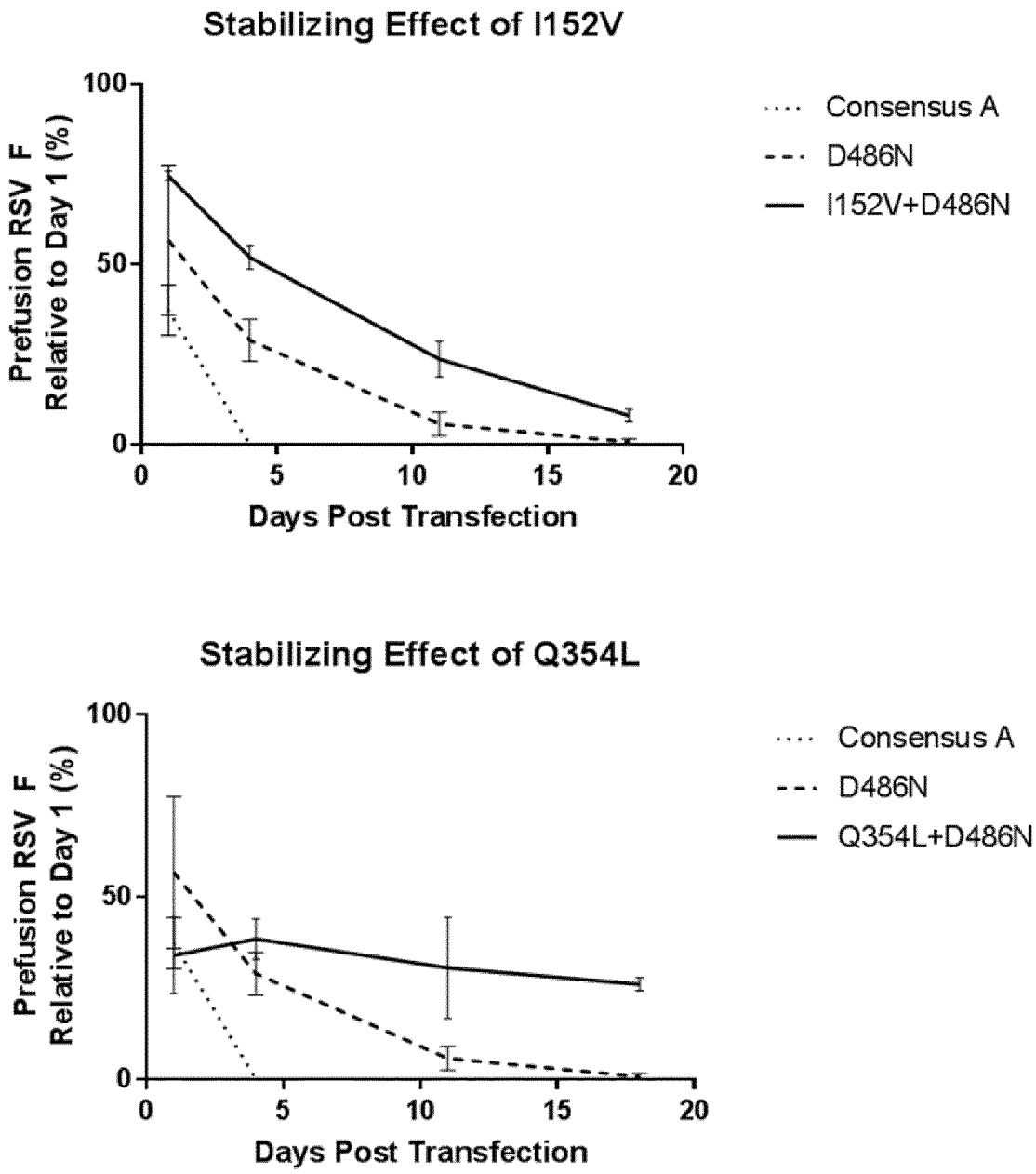
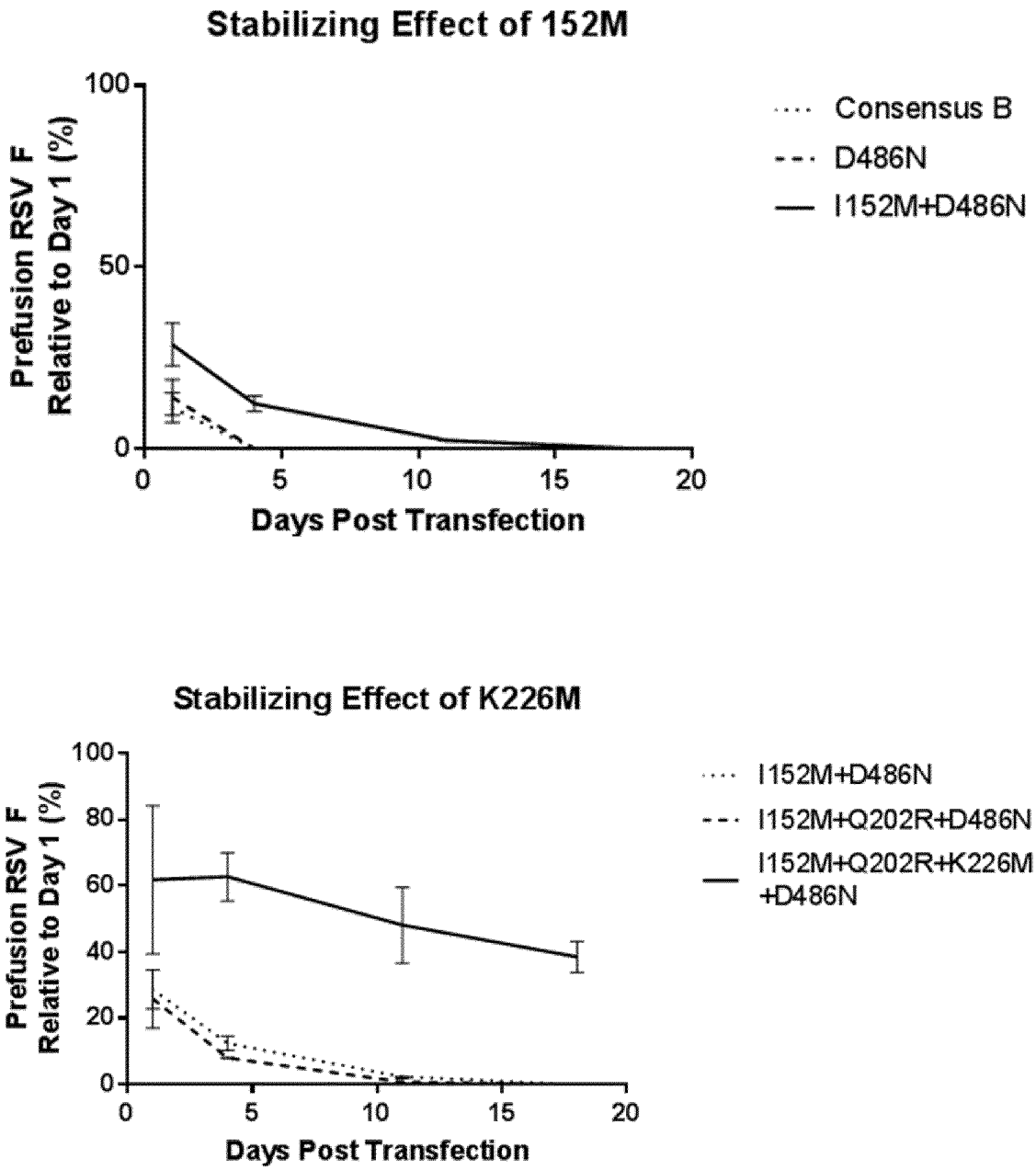



Fig. 3 - continued


Stabilizing Effect of 152M
Prefusion RSV F
Relative to Day 1 (%)
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20
Days Post Transfection
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D486N
I152M+D486N
Stabilizing Effect of K226M
Prefusion RSV F
Relative to Day 1 (%)
100
80
60
40
20
0
0
5
10
15
20
Days Post Transfection
I152M+D486N
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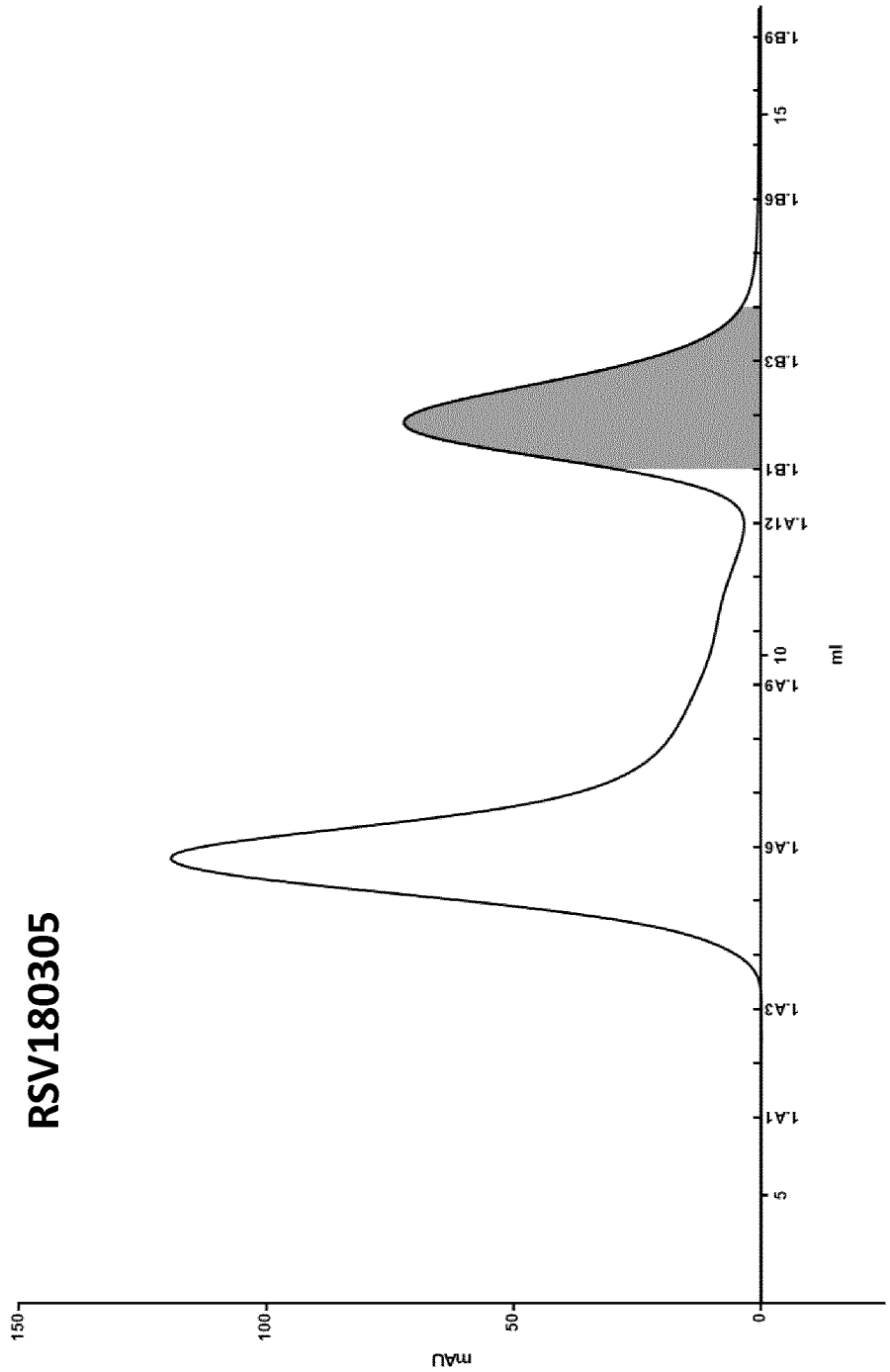
I152M+Q202R+K226M
+D486N

Fig. 4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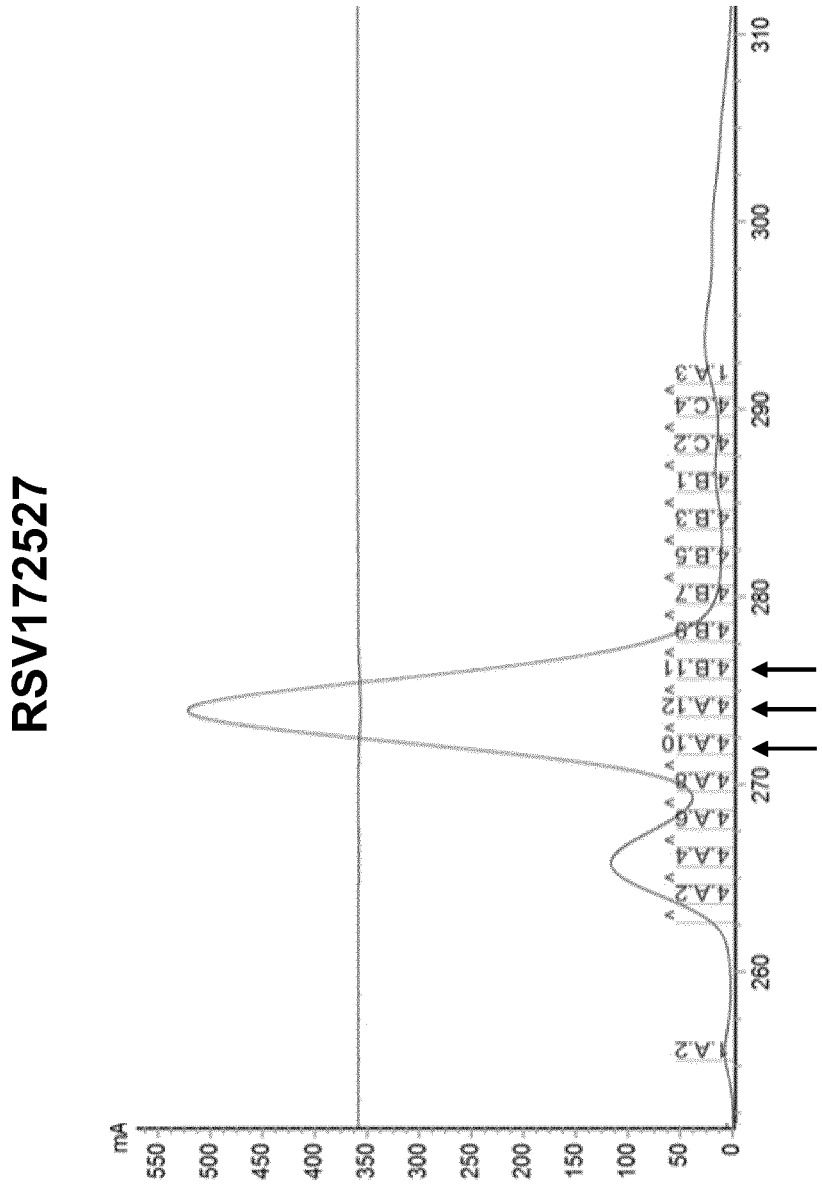
ml

Fig. 5A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310
1.A.2
4.A.2
4.A.4
4.A.6
4.A.8
4.A.10
4.A.12
4.B.11
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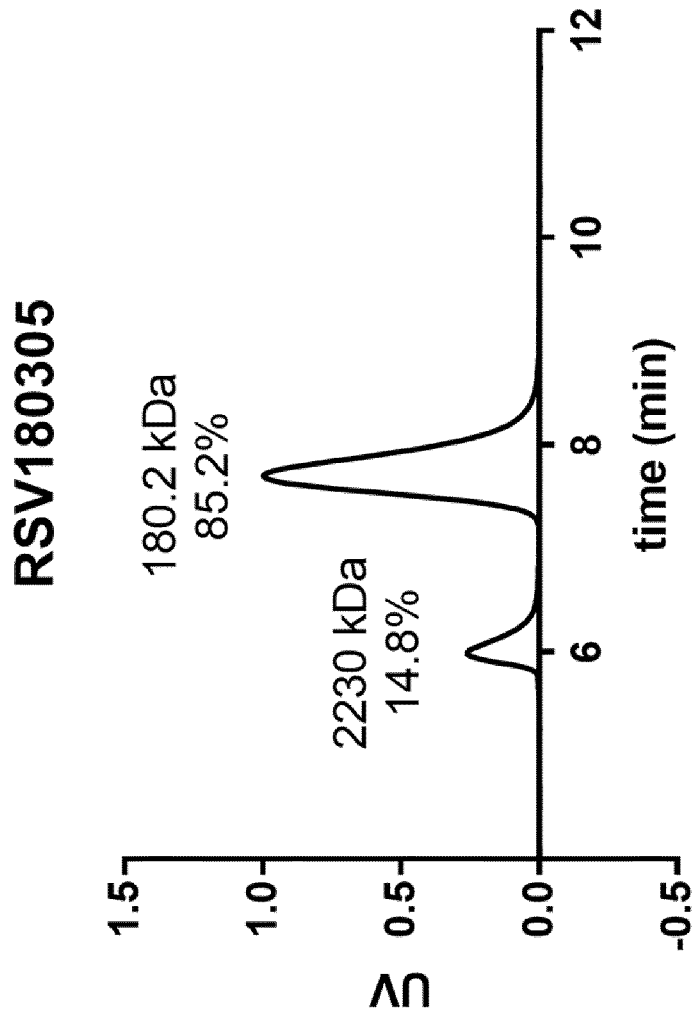
1.A.3

Fig. 5B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2230 kDa
14.8%
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8
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12
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UV

Fig. 5C

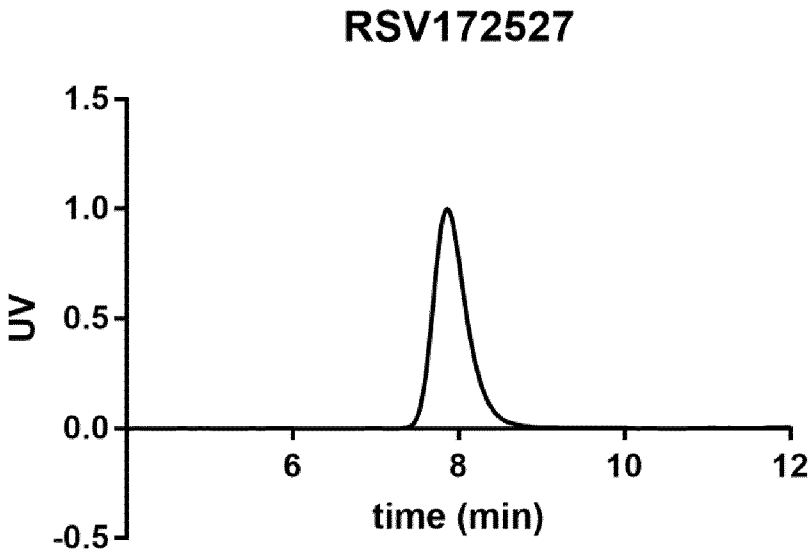



Fig. 5D

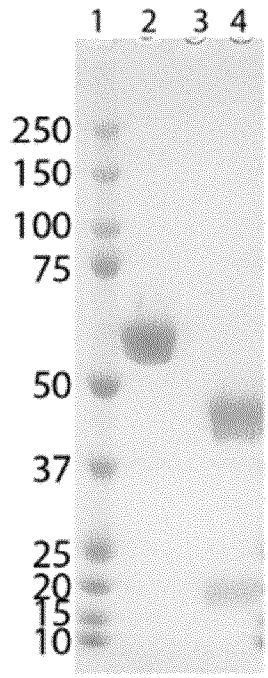
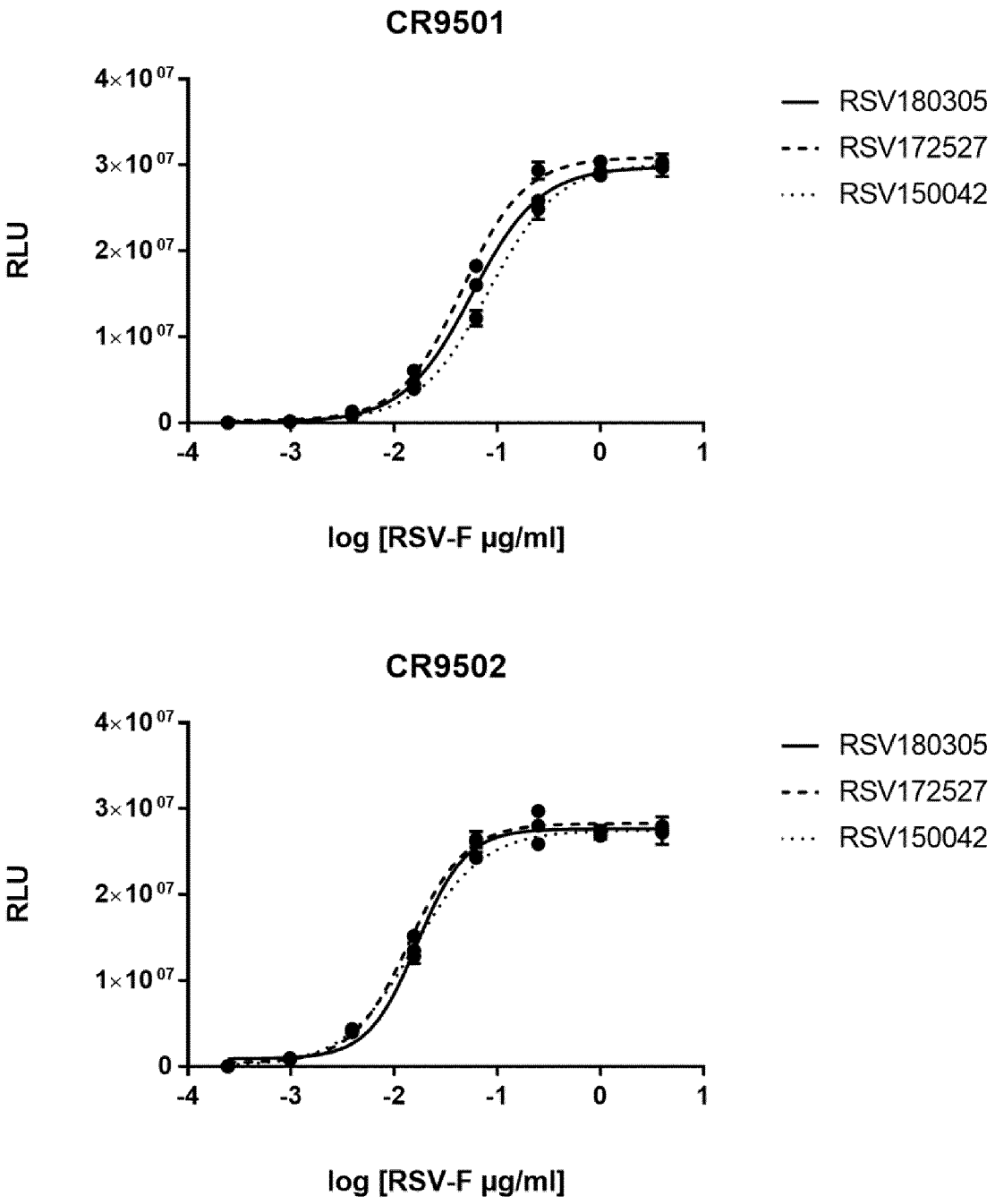



Fig. 6


CR9501
RLU
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3×10 07
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0
-4
-3
-2
-1
0
1
log [RSV-F µg/ml]
RSV180305
RSV172527
RSV150042
CR9502
RLU
4×10 07
3×10 07
2×10 07
1×10 07
0
-4
-3
-2
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0
1
log [RSV-F µg/ml]
RSV180305
RSV172527
RSV150042

Fig. 7

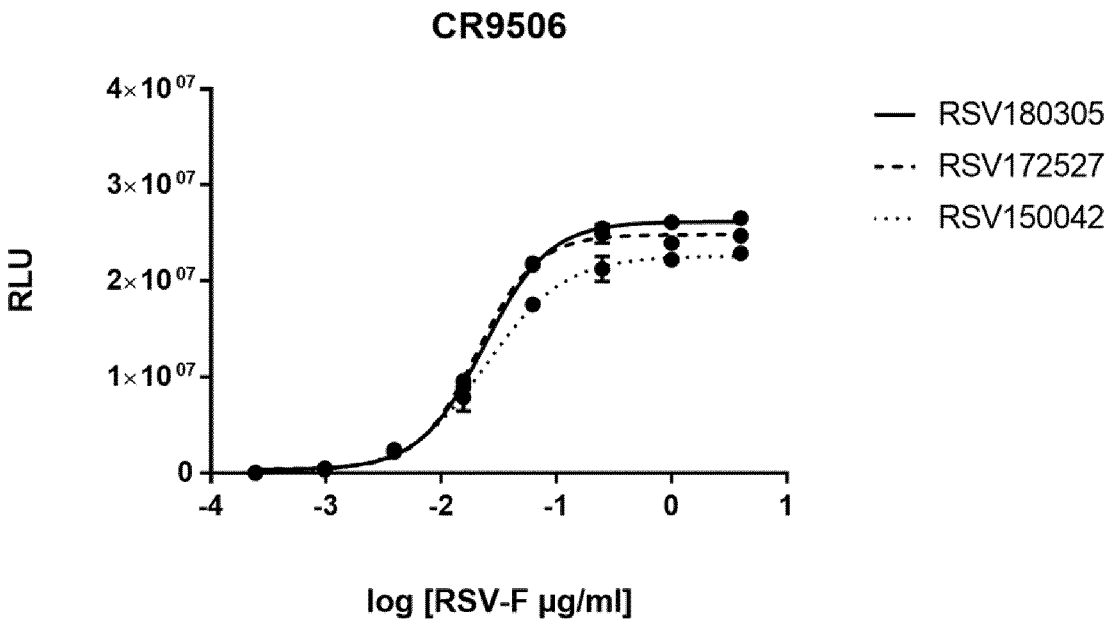
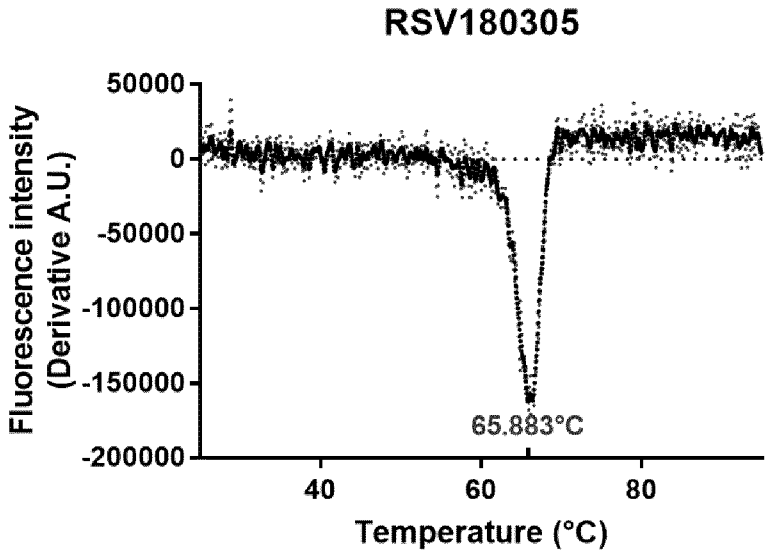



Fig. 7 - continued

A


RSV180305
Fluorescence intensity
(Derivative A.U.)
50000
0
-50000
-100000
-150000
-200000
65.883°C
40
60
80
Temperature (°C)

B

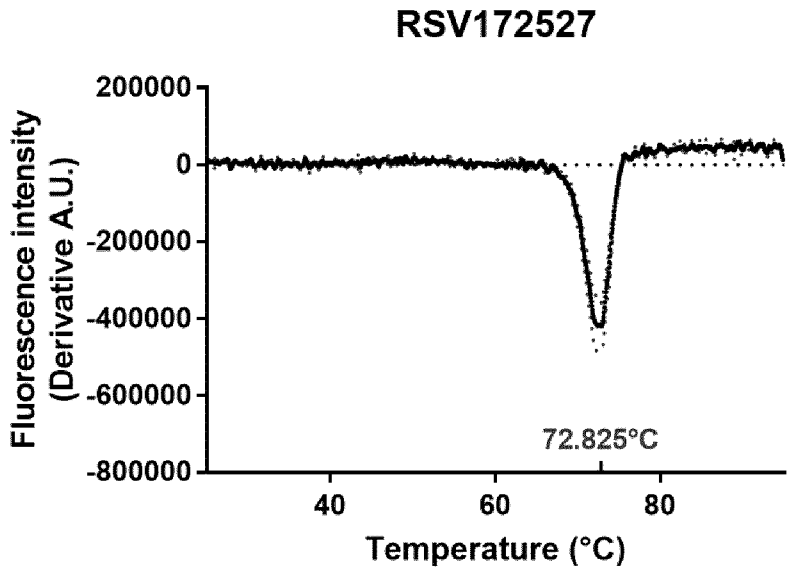

RSV172527
Fluorescence intensity
(Derivative A.U.)
200000
0
-200000
-400000
-600000
-800000
72.825°C
40
60
80
Temperature (°C)

Fig. 8