

Stabilized soluble pre-fusion RSV F polypeptides

The present invention relates to the field of medicine. The invention in particular relates to recombinant pre-fusion RSV F polypeptides and uses thereof, e.g. in immunogenic
5 compositions.

Background of the invention

Respiratory syncytial virus (RSV) is an enveloped non-segmented negative- strand RNA virus in the family Paramyxoviridae, genus Pneumovirus. Worldwide, it is estimated
10 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 immune-compromised 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 a peak of
15 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. The level of RSV disease burden, mortality and morbidity in the elderly are second only to those caused by non-pandemic influenza A infections.

To infect a host cell, RSV, like other enveloped viruses such as influenza virus and
20 HIV, require fusion of the viral membrane with a host cell membrane. For RSV the conserved fusion protein (RSV F protein) fuses the viral and host cell cellular membranes. In current models, based on paramyxovirus studies, the RSV F protein initially folds into a "pre-fusion" conformation. During cell entry, the pre-fusion conformation undergoes refolding and conformational changes to its "post-fusion" conformation. Thus, the RSV F protein is a
25 metastable protein that drives membrane fusion by coupling irreversible protein refolding to

membrane juxtaposition by initially folding into a metastable form (pre-fusion conformation) that subsequently undergoes discrete/stepwise conformational changes to a lower energy conformation (post-fusion conformation).

It is clear from electron microscopy of RSV-F that large structural differences between the pre-fusion and post-fusion F trimer exist, which has recently been confirmed by crystallography (McLellan J.S. et al. Science 340(6136):1113-7 (2013) and McLellan J.S. et al. Science 342(6158): 592-8 (2013)). These observation suggest that pre-fusion and post-fusion RSV F protein are antigenically distinct (Calder, L. J. et al. Virology 271, 122-131 (2000)).

A vaccine against RSV infection is not currently available, but is desired. Vaccine candidates based on the RSV F protein have failed due to problems with e.g. stability, purity, reproducibility, and potency. 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). However, these efforts have not yet yielded stable pre-fusion RSV F polypeptides that could be used as candidates for testing in humans.

Summary of the invention

The present invention provides stable, recombinant, pre-fusion respiratory syncytial virus (RSV) fusion (F) polypeptides, i.e. recombinant RSV F polypeptides that are stabilized in the pre-fusion conformation. The RSV F polypeptides of the invention comprise at least

one epitope that is specific to the pre-fusion conformation F protein. In certain embodiments, the pre-fusion RSV F polypeptides are soluble. In certain embodiments, the polypeptides are membrane-bound. The invention also provides nucleic acid molecules encoding the pre-fusion RSV F polypeptides according to the invention and vectors comprising such nucleic acid molecules.

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

Brief description of the Figures

FIG. 1: A) Superdex200 gel filtration chromatogram of the eluate A2_F24 N67I+S215P from the ion-exchange column. The arrows indicate the elution points of standard protein (1-Thyroglobulin 669 kDa, 2-Ferritin 440 kDa and 3-IgG 150 kDa). B) SDS-PAGE analysis of the pre-fusion F protein containing peak from the SEC chromatogram under reducing conditions.

FIG. 2: Western blot of NativePAGE loaded with samples containing 1) supernatant from cells expressing pre-fusion construct with the isoleucine zipper (S) F43; 2) supernatant from cells expressing mainly trimeric (top band) post-fusion RSV F protein; and 3) purified trimeric pre-fusion A2_F24 N67I.

- 5 FIG. 3: Expression levels of the point mutated constructs relative to the non-mutated A2_F24.

FIG. 4 shows the results of the method described in Example 6(A), determining the temperature where 50% of the CR9501 binding is lost; (B) shows a comparison of the stability of pre-fusion F (A2_F24 N67I+S215P) and the unmodified ectodomain when
10 assessed by 50% loss of binding of the pre-fusion-specific antibody CR9501.

FIG. 5: Octet measurements showing the storage-time dependent loss of binding of the pre-fusion-specific antibody CR9501 to the pre-fusion constructs; A) A2_F24 (SEQ ID NO: 19), B) A2_F24 K465Q, C) A2_F24 S46G, D) A2_F24 N67I and E) A2_F24 E92D at days 1, 5 and 33.

- 15 FIG. 6: Octet measurements showing the storage-time dependent loss of binding of the pre-fusion-specific monoclonal antibody CR9501 to the pre-fusion constructs; A) A2_F24 K465Q, B) A2_F24 K465Q+N67I, C) A2_F24 S46G, D) A2_F24 S46G+E92D, E) A2_F24 S46G+N67I, F) A2_F24 E92D, G) A2_F24 S46G+E92D, H) A2_F24 N67I+E92D and I) A2_F24 E92D+S215P at days 1, 5 and 33.

- 20 FIG. 7: VNA titers of mice at week 6 after a prime boost at week 0 and 4 with immunogens and doses according to Table 14.

FIG. 8: VNA titers of cotton rats at week 7 after a prime boost at week 0 and 4 with immunogens and doses according to Table 15.

FIG. 9: Lung and nose viral load at 5 days after i.n. RSV challenge.

Detailed description of the invention

5 The fusion protein (F) of the respiratory syncytial virus (RSV) is involved in fusion of the viral membrane with a host cell membrane, which is required for infection. The 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: 1) that is removed by a signal peptidase in the endoplasmic reticulum. F0 is cleaved at two sites
10 (between amino acid residues 109/110 and 136/137) by cellular proteases (in particular furin) in the trans-Golgi, 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
15 (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.

 A vaccine against RSV infection is not currently available, but is desired. One potential approach to producing a vaccine is a subunit vaccine based on purified RSV F
20 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, that is stable over time, and can be produced in sufficient quantities. In addition, for a subunit-based 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). Because the TM
25 region is responsible for membrane anchoring and trimerization, the anchorless soluble F

protein is considerably more labile than the full-length protein and will 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.

5 Stabilization of another paramyxovirus F protein in the pre-fusion conformation has been successfully accomplished for parainfluenza type 5 (PIV5). Yin et al. (Nature 439: 38-44 (2006)) thus stabilized the pre-fusion structure of PIV-5 F protein by mutation of the furin cleavage site in F₀ which blocked processing into F1 and F2. Furthermore, the transmembrane (TM) and cytoplasmic domain were replaced by a well-known helical
10 trimerization domain: GCN4pII. This domain forms a trimeric helical coiled coil structure and is a modification of the natural dimeric helical coiled coil peptide GCN4 (O'Shea et al., Science 243: 538-542 (1989)). The GCN4-pII peptide, in which the amino acid sequence of the GCN4 Leucine zipper was substituted with Isoleucine residues at every *a* and *d* position of the heptad, was shown to form a triple stranded parallel alpha-helical coiled coil (Harbury
15 et al., Science 262: 1401-1407 (1993)).

For the stabilization of RSV F in the pre-fusion conformation, the same strategy has been tried, such as e.g., mutation of the furin cleavage site, and fusion of the RSV-F ectodomain to a GCN4pII trimerization domain (as disclosed in e.g. WO2010/149743, WO2010/149745, WO2009/079796, WO2012/158613) or to the fibrin trimerization domain
20 (McLellan et al., Nature Struct. Biol. 17: 2-248-250 (2010)). This fibrin domain or 'Foldon' is derived from T4 fibrin and was described earlier as an artificial natural trimerization domain (Letarov et al., Biochemistry Moscow 64: 817-823 (1993); S-Guthe et al., J. Mol. Biol. 337: 905-915. (2004)). However, these efforts did not result in stable pre-fusion RSV-F protein. Moreover, these efforts have not yet resulted in candidates suitable for testing in
25 humans.

The present invention now provides recombinant stable pre-fusion RSV F polypeptides, i.e. RSV F polypeptides that are stabilized in the pre-fusion conformation. In the research that led to the present invention, several modification steps were introduced and/or combined in order to obtain said stable soluble pre-fusion RSV F polypeptides. The stable pre-fusion RSV F polypeptides of the invention 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 presented 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 polypeptides of the invention 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: 54, a heavy chain CDR2 region of SEQ ID NO: 55, a heavy chain CDR3 region of SEQ ID NO: 56 and a light chain CDR1 region of SEQ ID NO: 62, a light chain CDR2 region of SEQ ID NO: 63, and a light chain CDR3 region of SEQ ID NO: 64 (hereafter referred to as CR9501) and/or a pre-fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ ID NO: 58, a heavy chain CDR2 region of SEQ ID NO: 59, a heavy chain CDR3 region of SEQ ID NO: 60 and a light chain CDR1 region of SEQ ID NO: 66, a light chain CDR2 region of SEQ ID NO: 67, and a light chain CDR3 region of SEQ ID NO: 68 (referred to as CR9502). 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).

In certain embodiments, the recombinant pre-fusion RSV F polypeptides comprise at least one epitope that is recognized by at least one pre-fusion specific monoclonal antibody as described above and are trimeric.

In certain embodiments, the stable pre-fusion RSV F polypeptides according to the invention comprise a mutation of the amino acid residue on position 67 and/or a mutation of the amino acid residue on position 215.

In certain embodiments, the amino acid on position 67 is mutated to a hydrophobic amino acid.

In certain embodiments, the stable pre-fusion RSV F polypeptides according to the invention comprise a mutation of the amino acid residue N or T on position 67 and/or a mutation of amino acid residue S on position 215.

In certain embodiments, the stable pre-fusion RSV F polypeptides according to the invention comprise a F1 domain and a F2 domain, and a linking sequence comprising from 1 to 10 amino acid residues, linking said F1 domain to said F2 domain, wherein the polypeptides further comprise a mutation of the amino acid residue N or T on position 67 and/or a mutation of amino acid residue S on position 215.

In certain embodiments, the stable pre-fusion RSV F polypeptides according to the invention comprise a truncated F1 domain and a F2 domain, and a linking sequence comprising from 1 to 10 amino acid residues, linking said truncated F1 domain to said F2 domain, wherein the polypeptides further comprise a mutation of the amino acid residue N or T on position 67 and/or a mutation of amino acid residue S on position 215.

The polypeptides of the invention thus comprise at least one stabilizing mutation in the F1 and/or F2 domain as compared to the RSV F1 and/or F2 domain in a wild-type RSV F protein.

In certain embodiments, the pre-fusion RSV F polypeptides comprise a mutation of amino acid residue N or T on position 67 (N/T67I) into I and/or a mutation of amino acid residue S on position 215 into P (S215P).

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 the sequence of RSV F protein from the A2 strain (SEQ ID NO: 1). 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 the RSV A2 strain of SEQ ID NO: 1. 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: 1) When a RSV strain other than the A2 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 the A2 strain of SEQ ID NO: 1 by aligning the sequences of the other RSV strain with the F protein of SEQ ID NO: 1 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.

An amino acid according to the invention can be any of the twenty naturally occurring (or ‘standard’ amino acids) or variants thereof, such as e.g. D-amino acids (the D-enantiomers of amino acids with a chiral center), or any variants that are not naturally found in proteins, such as e.g. norleucine. 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

polypeptide backbone, and glycine that is more flexible than other amino acids. Table 11 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
5 result in increased expression levels and/or increased stabilization of the pre-fusion RSV F polypeptides as compared RSV F polypeptides that do not comprise these mutation(s).

In certain embodiments, the pre-fusion RSV F polypeptides are soluble.

In certain embodiments, the pre-fusion RSV F polypeptides further comprise a heterologous trimerization domain linked to said truncated F1 domain. According to the
10 invention, it was shown that by linking a heterologous trimerization domain to the C-terminal amino acid residue of a truncated F1 domain, optionally combined with a linking sequence linking the F1 and F2 domain, and the stabilizing mutation(s), RSV F polypeptides are provided that show high expression and that bind to pre-fusion-specific antibodies, indicating that the polypeptides are in the pre-fusion conformation. In addition, the RSV F polypeptides
15 are stabilized in the pre-fusion conformation, i.e. even after processing of the polypeptides they still bind to the pre-fusion specific antibodies CR9501 and/or CR9502, indicating that the pre-fusion specific epitope is retained.

In further embodiments, the pre-fusion RSV F polypeptides comprise one or more further mutations (as compared to the wild-type RSV F protein), selected from the group
20 consisting of:

- (a) a mutation of the amino acid residue on position 46;
- (b) a mutation of the amino acid residue on position 77;
- (c) a mutation of the amino acid residue on position 80;
- (d) a mutation of the amino acid residue on position 92;
- 25 (e) a mutation of the amino acid residue on position 175;

- (f) a mutation of the amino acid residue on position 184;
- (g) a mutation of the amino acid residue on position 185;
- (h) a mutation of the amino acid residue on position 201;
- (i) a mutation of the amino acid residue on position 209;
- 5 (j) a mutation of the amino acid residue on position 421;
- (k) a mutation of the amino acid residue on position 426;
- (l) a mutation of the amino acid residue on position 465;
- (m) a mutation of the amino acid residue on position 486;
- (n) a mutation of the amino acid residue on position 487; and
- 10 (o) a mutation of the amino acid residue on position 508.

In preferred embodiments, the one or more further mutations are selected from the group consisting of:

- (a) a mutation of the amino acid residue S on position 46 into G (S46G);
- (b) a mutation of the amino acid residue K on position 77 into E (K77E);
- 15 (c) a mutation of the amino acid residue K on position 80 into E (K80E);
- (d) a mutation of the amino acid residue E on position 92 into D (E92D);
- (e) a mutation of the amino acid residue N on position 175 into P (N175P);
- (f) a mutation of the amino acid residue G on position 184 into N (G184N);
- (g) a mutation of the amino acid residue V on position 185 into N (V185N);
- 20 (h) a mutation of the amino acid residue K on position 201 into Q (K201Q);
- (i) a mutation of the amino acid residue K on position 209 into Q (K209Q);
- (j) a mutation of the amino acid residue K on position 421 into N (K421N);
- (k) a mutation of the amino acid residue N on position 426 into S (N426S);
- (l) a mutation of the amino acid residue K on position 465 into E or Q (K465Q);
- 25 (m) a mutation of the amino acid residue D on position 486 into N (D486N);

(n) a mutation of the amino acid residue E on position 487 into Q, N or I
(E487Q/N/I); and

(o) a mutation of the amino acid residue K on position 508 into E (K508E).

It is again noted that for the positions of the amino acid residues reference is made to

5 SEQ ID NO: 1. A skilled person will be able to determine the corresponding amino acid residues in F proteins of other RSV strains.

In certain embodiments, the pre-fusion RSV F polypeptides comprise at least two mutations (as compared to a wild-type RV F protein). In preferred embodiments the at least two mutations are a mutation of the amino acid N or T on position 67 into I (N/T67I) and a
10 mutation of the amino acid S on position 215 into P (S215P).

In certain embodiments, the pre-fusion RSV F polypeptides comprise at least one further mutation, selected from the group consisting of:

- (a) a mutation of the amino acid residue S on position 46 into G;
- (b) a mutation of the amino acid residue K on position 77 into E;
- 15 (c) a mutation of the amino acid residue K on position 80 into E;
- (d) a mutation of the amino acid residue E on position 92 into D;
- (e) a mutation of the amino acid residue N on position 175 into P;
- (f) a mutation of the amino acid residue G on position 184 into N;
- (g) a mutation of the amino acid residue V on position 185 into N;
- 20 (h) a mutation of the amino acid residue K on position 201 into Q;
- (i) a mutation of the amino acid residue K on position 209 into Q;
- (j) a mutation of the amino acid residue K on position 421 into N;
- (k) a mutation of the amino acid residue N on position 426 into S;
- (l) a mutation of the amino acid residue K on position 465 into E or Q;
- 25 (m) a mutation of the amino acid residue D on position 486 into N;

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

(o) a mutation of the amino acid residue K or R on position 508 into E.

In certain embodiments, the polypeptides comprise at least three mutations.

In certain embodiments, the heterologous trimerization domain comprises the amino acid sequence EKKIEAIEKKIEAIEKKIEA (SEQ ID NO: 3). In certain other embodiments, the heterologous trimerization domain comprises the amino acid sequence GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 4).

As described above, in certain embodiments the polypeptides of the invention 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 other embodiments, the F1 domain is truncated after amino acid residue 495 of the RSV F protein (referring to SEQ ID NO: 1), i.e. the C-terminal part of the F1 domain starting from amino acid residue 496 (referring to SEQ ID NO: 1) has been deleted. In certain other embodiments, the F1 domain is truncated after amino acid residue 513 of the RSV F protein. In certain embodiments, the F1 domain is truncated after amino acid residue 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, 501, 502, 503, 504, 505, 506, 507, 508, 509, 510, 512, 513, 514, 515, 516, 517, 518, 519, 520, 521, 522, 523, 525 or 525.

In certain embodiments, the trimerization domain is linked to amino acid residue 495 of the RSV F1 domain. In certain embodiments, the trimerization domain comprises SEQ ID NO: 4 and is linked to amino acid residue 495 of the RSV F1 domain.

In certain other embodiments, the trimerization domain is linked to amino acid residue 513 of the RSV F1 domain. In certain embodiments, the trimerization domain comprises SEQ ID NO: 3 and is linked to amino acid residue 513 of the RSV F1 domain.

In certain embodiments, the F1 domain, which is optionally truncated, and the F2 domain are linked by a linking sequence, linking the C-terminal amino acid of the F2 domain to the N-terminal amino acid of the (optionally truncated) F1 domain. In certain embodiments, the linking sequence (or linker) comprises from 1-10 amino acid residues, preferable from 2-9 amino acid residues, preferably from 3-8 amino acid residues, preferably from 4-7 amino acid residues, more preferably the linker comprises 5 or 6 amino acid residues. Numerous conformationally neutral linkers are known in the art that can be used according to the invention without disrupting the conformation of the pre-fusion RSV F polypeptides. In preferred embodiments, the linker comprises the amino acid sequence GSGSG (SEQ ID NO: 5).

In certain embodiments, the F1 domain and/or the F2 domain are from an RSV A strain. In certain embodiments the F1 and/or F2 domain are from the RSV A2 strain of SEQ ID NO: 1.

In certain embodiments, the F1 domain and/or the F2 domain are from an RSV A strain are from the RSV A strain of SEQ ID NO: 69.

In certain embodiments, the F1 domain and/or the F2 domain are from an RSV B strain. In certain embodiments the F1 and/or F2 domain are from the RSV B strain of SEQ ID NO: 2.

In certain embodiments, the F1 and F2 domain are from the same RSV strain. In certain embodiments, the pre-fusion RSV F polypeptides are chimeric polypeptides, i.e. comprising F1 and F2 domains that are from different RSV strains.

In certain embodiments, the level of expression of the pre-fusion RSV F polypeptides of the invention is increased, as compared to a wild-type RSV F polypeptide ectodomain (i.e.

without the transmembrane and cytoplasmic region) without the mutation(s). In certain embodiments the level of expression is increased at least 5-fold, preferably up to 10-fold. In certain embodiments, the level of expression is increased more than 10-fold.

The pre-fusion RSV F polypeptides according to the invention are stable, i.e. do not readily change into the post-fusion conformation upon processing of the polypeptides, such as e.g. purification, freeze-thaw cycles, and/or storage etc.

In certain embodiments, the pre-fusion RSV F polypeptides according to the invention have an increased stability upon storage at 4°C as compared to a RSV F polypeptide without the mutation(s). In certain embodiments, the polypeptides are stable upon storage at 4°C for 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 polypeptides still display the at least one epitope specific for the a pre-fusion specific antibody (e.g. CR9501) upon storage of the polypeptide in solution (e.g. culture medium) at 4° C for at least 30 days, e.g. as determined using a method as described in Example 7 or 9. In certain embodiments, the polypeptides 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 polypeptides at 4°C.

In certain embodiments, the pre-fusion RSV F polypeptides according to the invention have an increased stability when subjected to heat, as compared to RSV F polypeptides without said mutation(s). In certain embodiments, the pre-fusion RSV F polypeptides are heat stable for at least 30 minutes at a temperature of 55° C, preferably at 58° C, more preferably at 60° C. With “heat stable” it is meant that the polypeptides still display the at least one pre-fusion specific epitope after having been subjected for at least 30 minutes to an increased temperature (i.e. a temperature of 55°C or above), e.g. as determined using a method as described in Example 6.

In certain embodiments, the polypeptides 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 preferred embodiments, the pre-fusion RSV F polypeptide of the invention
5 comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 21-52 and 71-89. In certain embodiments, the pre-fusion RSV F polypeptide of the invention consists of an amino acid sequence selected from the group consisting of SEQ ID NO: 21-52 and 71-89.

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

In certain embodiments, the encoded polypeptides according to the invention further comprise a leader sequence, also referred to as signal sequence or signal peptide, corresponding to amino acids 1-26 of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 69. This
15 is a short (typically 5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway. In certain embodiments, the polypeptides according to the invention do not comprise a leader sequence.

In certain embodiments, the polypeptides comprise a HIS-Tag. A His-Tag or polyhistidine-tag is an amino acid motif in proteins that consists of at least five histidine (H)
20 residues, often at the N- or C-terminus of the protein, which is generally used for purification purposes.

In certain embodiments, the polypeptides do not comprise a HIS-Tag. According to the invention, it has surprisingly been shown that when the HIS-tag is deleted the level of expression and the stability are increased as compared to polypeptides with a HIS-tag.

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

In preferred embodiments, the nucleic acid molecules encoding the polypeptides 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 polypeptide 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 polypeptide sequence encoded by the nucleic acid molecules to reflect the codon usage of any particular host organism in which the polypeptides 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).

5 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
10 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, such as e.g. Ad26 or Ad35, alphavirus, paramyxovirus, vaccinia virus, herpes virus, retroviral
15 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 polypeptides form also part of the invention. The pre-fusion RSV F polypeptides may be produced through recombinant DNA technology involving expression of the molecules in
20 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
25 cells. In general, the production of a recombinant proteins, such the pre-fusion RSV F

polypeptides of the invention, in a host cell comprises the introduction of a heterologous nucleic acid molecule encoding the polypeptide 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 polypeptide 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 polypeptides. 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
5 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
10 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
15 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 polypeptide and/or a nucleic acid molecule, and/or a vector, as described above. The
20 invention thus provides compositions comprising a pre-fusion RSV F polypeptide 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 polypeptide. The invention further provides immunogenic compositions comprising a pre-fusion RSV F
25 polypeptide, and/or a nucleic acid molecule, and/or a vector, as described above. The

invention also provides the use of a stabilized pre-fusion RSV F polypeptide, 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 polypeptide, and/or a nucleic acid molecule, and/or a vector, according to the invention. Also provided are pre-fusion RSV F polypeptides, 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 polypeptides, 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 polypeptides, 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. ≥ 50 years old, ≥ 60 years old, and preferably ≥ 65 years old), the young (e.g. ≤ 5 years old, ≤ 1 year old), hospitalized patients and patients who have been treated with an antiviral compound but have shown an inadequate antiviral response.

The pre-fusion RSV F polypeptides, 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 polypeptides, 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 polypeptide, nucleic acid molecule and/or a vector, as described above. A therapeutically effective amount refers to an amount of a polypeptide, 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 polypeptide, 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 polypeptides, 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 polypeptides 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 polypeptides may be formulated into an injectable preparation.

In certain embodiments, a composition according to the invention further comprises

5 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 polypeptides of the invention. Examples of suitable adjuvants include

10 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

15 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

20 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 polypeptides may also be administered in combination with or

25 conjugated to nanoparticles, such as e.g. polymers, liposomes, virosomes, virus-like particles.

The pre-fusion F polypeptides 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.

5 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 a composition 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
10 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 polypeptide and/or a nucleic acid molecule encoding a pre-fusion RSV F polypeptide, and/or
15 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
20 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 polypeptides in a composition for a single administration can for instance be
5 about 0.01 μ g to about 10 mg, e.g. 1 μ g – 1 mg, e.g. 10 μ g – 100 μ g. Determining the recommended dose will be carried out by experimentation and is routine for those skilled in the art.

Administration of the compositions according to the invention can be performed using standard routes of administration. Non-limiting embodiments include parenteral
10 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.

15 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 polypeptides, 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
20 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 polypeptides of the invention may be used as diagnostic tool, for
25 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 polypeptide 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 polypeptide according to the invention; and b) detecting the presence of antibody-polypeptide complexes.

The invention further provides a method for stabilizing the pre-fusion conformation of an RSV F polypeptide, comprising introducing one or more mutations in a RSV F1 and/or F2 domain, as compared to the wild-type RSV F1 and/or F2 domain, wherein the one or more mutations are selected from the group consisting of:

- 10 (a) a stabilizing mutation that locks the HRA domain from hinging in a region adjacent to the conserved 69-212 disulfide bridge, said region comprising the amino acid residues 66-68 and 214-216,
- (b) a mutation in the helix (at the C-terminus of the F2 domain) comprising the amino acid residues 76-98 at the C-terminus of the F2 domain;
- 15 (c) a mutation that reduces the negative charge repulsion between the top of the HRB stem region (N-terminal end of HRB) comprising amino acids 486, 487 and 489; and
- (d) a stabilizing mutation in the HRA region.

In certain embodiments, the mutation in the HRA hinge region is at position 67.

20 In certain embodiments, the mutation in the HRA hinge region is at position 215.

In certain embodiments, the mutation in the HRA hinge region is at position 66 or 68, and/or at position 214 or 216.

In certain embodiments, the mutation in the helix is at position 77.

In certain embodiments, the mutation in the helix is at position 80.

In certain embodiments, the amino acid residue at position 77 and/or 80 is changed into a negatively charged amino acid.

In certain embodiments, the mutation is at position 92.

In certain embodiments, the mutation that reduces the negative charge repulsion between the top of the HRB stem region comprising amino acids 486, 487, 489.

In certain embodiments, the mutation is at position 489.

In certain embodiments, the mutation is at position 486.

In certain embodiments, the mutation stabilizes the beta-turns between the amino acid residues 175-193.

In certain embodiments, the mutation is stabilizing the turn at position 175.

In certain embodiments, the mutation is stabilizing the turn at position 184 -185.

Stabilized pre-fusion RSV F polypeptides obtainable and/or obtained by such method also form part of the invention, as well as the uses thereof as described above.

The invention is further explained in the following examples. The examples do not limit the invention in any way. They merely serve to clarify the invention.

Examples

EXAMPLE 1

Preparation of stable pre-fusion RSV F polypeptides – linkers and trimerization domains

In the research that led to the present invention, stabilized variants of soluble pre-fusion F protein (sF) were designed by stabilizing the two main regions that initiate refolding.

The first strategy was to arrest the fusion peptide in its position and prevent it from getting

released from the head region by fixing and joining the F1-F2 domains by a short loop.

linker was designed based on the distances measured in a 3D homology model that was generated for RSV-F type A2 based on the sequence alignment with the F sequence of parainfluenza type 5 for which a 3D structure is published (Yin et. al., 2006).

	AAA47881PIV5	FIVVKLMPTIDSPISGCNI--TSISSYNATVTKLLQPIGENLETIRNQLIP--TRRRRR-
15	FUS_HRSV1B	SVITIELSNIKET--KCNGTDTKVKLKIQELDKYKNAVTELQLLMQNTPAANNRAREAP
	ACO83301HRSVA2ref	SVITIELSNIKKN--KCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARELP
		:.. :..*.. ** :.. : : * :: * :.. . **.

	AAA47881PIV5	KTNAAVADVQATQSLGTAVQAVQDHSVSPAITAANCKAQDAIIGSILNLYLTELT
25	FUS_HRSV1B	STNKAVVSLSNGVSVLTSKVLDLKNIYINNRLLPVNNQQSCRISNIETVIEFQQMNSRLLE
	ACO83301HRSVA2ref	STNKAVVSLSNGVSVLTSKVLDLKNIYIDKQLLPVNNKQSCSISNIETVIEFQQKNNRLLE
		. ** * * . . : : . . . * : * : : : * : . : * : . . * . : : . . *

	AAA47881PIV5	IFHNQITNP-ALSPITIQALRILLGSTLPTVVEKSFNTQISAAELLSSGLLTGQIVGLDL
30	FUS_HRSV1B	ITREFSVNAGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSII
	AC083301HRSVA2ref	ITREFSVNAGVTTVPSTYMLTNSELLSLINDMPITNDQKKLMSSNNVQIVRQQSYSIMSII

		* :: .*. . :*: * :* . : : : : . : :
	AAA47881PIV5	TYMQMVIKIELPTLTVQPATQIIDLATISAFI--NNQEVMAQL--PTRVMVTG--SLIQA
	FUS_HRSV1B	KEEVLAYVVQLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRDRGWYCDNAGSVSFF
5	ACO83301HRSVA2ref	KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRDRGWYCDNAGSVSFF
		. :. :*: * :. :. :. :. : *
	AAA47881PIV5	YPASQCTITPNTVYCRYNDAQVLSDDTMACLQGN--LTRCTFSPVVGSLTRFVLFEDI
	FUS_HRSV1B	PQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLG-
10	ACO83301HRSVA2ref	PQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLG-
		*. *. :. * *: :. *. :. * . *. :. . . : *
	AAA47881PIV5	VYANCRS-MLCKCMQPAAVILQPSSSPVTVIDMYKCVSLQLDNLRFITITQLANVTYNSTI
	FUS_HRSV1B	AIVSCYGKTKCTASNKNRGI IKTFSNGCDYVSNKGVDTVSVGNTLYYVNKLEGKNLYVKG
15	ACO83301HRSVA2ref	AIVSCYGKTKCTASNKNRGI IKTFSNGCDYVSNKGVDTVSVGNTLYYVNKQEGKSLYVKG
		. ..* . *.. : *:. *. :. :. :.* : :. . .
	AAA47881PIV5	KLESS--QILSIDPLDISQNLAAVNKSLSDALQHQAQSDTYLSAITSATTTT--VLSIIA
	FUS_HRSV1B	EPIINYYDPLVFPSEDFDASISQVNEKINQSLAFIRRSDELLHNVNTGKSTTNIMITTII
20	ACO83301HRSVA2ref	EPIINFYDPLVFPSEDFDASISQVNEKINQSLAFIRKSDDELLHNVNAVSTTNIMITTII
		: . : * : . :. :. :*: :. :* :. :* * :. :*: :. : *
	AAA47881PIV5	ICLGSLGLILIIILLSVVVWKLITIVVANRNRMENFVYHK-----
	FUS_HRSV1B	IVIIIVLLSLIAIGLLLYCKAKNTPVTLTKDQLSGINNIAFSK-----
25	ACO83301HRSVA2ref	IVIIIVLLSLIAVGLLLYCKARSTPVTLTKDQLSGINNIAFSN-----
		* : : * ** : :. * . *: : . : :

Alignment between F sequence of HRSV type A and B with PIV5 (top sequence) that was used to construct the homology model of prefusion RSV-F

The other unstable region is the second heptad repeat (HRB) region that forms the trimeric helical stem region in pre-fusion F protein. Deletion of the transmembrane domain

(TM) in the soluble F protein further destabilizes this region, which was compensated by the addition of different heterologous trimerization domains. The fully processed mature RSV-F ectodomain was fused C-terminally to different trimerization domains and at different positions (i.e. the F1 domain was truncated at different amino acid residues).

5 Several constructs were made based on either RSV A2 or B1 strains. Different trimerization domains were linked to the RSV F1 domain, which was truncated at different positions. Trimerization domains that were tested included the Fibrin motif (comprising the amino acid sequence: GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 4), and the “Fibrin long” motif, a longer, N-terminal extended Fibrin domain which includes its
10 natural helical regions (comprising the amino acid sequence: SSLQGDVQALQEAGYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 6), that were added to the RSV F1 domain in frame (in register) with the presumed heptad repeat of the HRB region.

 Further constructs that were made comprised heptad ideal helical trimeric coiled coils,
15 or Isoleucine Zipper domains (IZ) (Suzuki et al., Protein Engineering 11: 1051-1055 (1998)), comprising the amino acid sequence: IEAIEKK (SEQ ID NO: 7). According to the invention different IZ domains were used, referred to as Isoleucine Zipper (L), comprising the amino acid sequence: (I)EKKIEAIEKKIEAIEKKIEAIEAIEKKIEA (SEQ ID NO: 8) and Isoleucine Zipper (S), comprising the amino acid sequence EKKIEAIEKKIEAIEKKIEA (SEQ ID NO:
20 3).

 These IZ domains are comparable in structure to GCN4, however, the IZ domains are not natural sequences but designed to be optimal trimerization domains and therefore more stable.

 Further constructs were made with other known trimerization domains:

GCN4II

EDKIEEILSKIYHIENEIARIKKLIGEA (SEQ ID NO: 9)

Optimized GCN4II

EDKVEELLSKIYHIENRIARIEKLVGEA (SEQ ID NO: 10)

5 **Matrilin -1 (long version)**

EEDPCECKSIVKFQTKVEELINTLQQKLEAVAKRIEALenkII (SEQ ID NO: 11)

Matrillin- 1 short version that only contains zipper domain:

EELINTLQQKLEAVAKRIEALenkII (SEQ ID NO: 12)

10 The following constructs were made:

Construct F18 comprised the Fibrin trimerization domain (SEQ ID NO: 4) linked to amino acid residue 513 of the F1 domain.

Construct F19 comprised the Fibrin trimerization domain (SEQ ID NO: 4) linked to amino acid residue 499 of the F1 domain.

15 Construct F20 comprised the Isoleucine Zipper (L) domain (SEQ ID NO: 8) linked to amino acid residue 516 of the F1 domain and comprising additional modifications in HRB to optimize the hydrophobic nature of the heptad positions and facilitate in-frame fusion with the IZ domain.

Construct F21 also comprised Isoleucine Zipper (L) domain (SEQ ID NO: 8), but linked to
20 amino acid residue 501 of the F1 domain and without additional modifications in the HRB region.

Construct F22 comprised the Isoleucine Zipper (L) domain (SEQ ID NO: 8) linked to amino acid residue 495 of the F1 domain and comprising additional modifications in HRB.

Construct F23 comprised the Isoleucine Zipper (S) domain (SEQ ID NO: 3) linked to amino
25 acid residue 495.

Construct F46 also comprised the Isoleucine Zipper (S) domain (SEQ ID NO: 3) but linked to a longer RSV-F ectodomain, i.e. the F1 domain was truncated after amino acid residue 513.

All constructs comprised a HIS-tag.

The constructs were tested for expression levels, storage stability and antibody

5 binding with the antibody CR9501. The amino acid sequences of the heavy and light chain variable regions, and of the heavy and light chain CDRs of this antibody are given below.

CR9501 comprises the binding regions of the antibodies referred to as 58C5 in WO2012/006596.

The constructs were synthesized and codon-optimized at Gene Art (Life

10 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 293Freestyle cells (Life Technologies). The cells were transiently transfected using 293Fectin (Life Technologies) according to the manufacturer's instructions and cultured for 5 days at 37°C and 10% CO₂.

15 The culture supernatant was harvested and spun for 5 min at 300 g to remove cells and cellular debris. The spun supernatant was subsequently sterile filtered using a 0.22 µm vacuum filter and stored at 4°C until use.

Supernatants from day 5 were evaluated for F protein expression by western blot using the monoclonal antibody CR9503, which comprises the heavy and light chain variable

20 regions of the RSV F antibody Motavizumab (referred to as CR9503). The approximate expression levels of the pre-fusion RSV F protein constructs were assessed using CR9503, an anti-human IR-dye conjugated secondary antibody (Li-Cor, Lincoln, NE) or a HRP

conjugated mouse anti-human IgG (Jackson ImmunoResearch, West Grove, PA). The protein quantities were then estimated using a dilution series of purified RSV standard protein, either

25 by eye or using the Odyssey CLx infrared imaging system. Alternatively, Quantitative Octet

(BioLayer Interferometry) was used for measuring protein concentration in the Supernatants.

To evaluate construct stability and to identify positive or negative stabilizing effects of introduced trimerization motifs, the constructs capable of binding CR9501 were treated at a range of temperatures from 45-65 °C for 30 minutes to test the stability of the CR9501

5 epitope. This procedure is described in detail in Example 8. The results are summarized in Table 1.

Table 1. Expression and stability of RSV F constructs with different trimerization motifs

RSV Protein	Description				Stability*
	Trimerization motif	Modifications	Termination point	Expression (ug/ml)	
F18	Fibritin	None	513	2	unstable
F19	Fibritin	None	499	0	ND
F20	Isoleucine zipper (L)	502 509 516 Ile	516	0	ND
F21	Isoleucine zipper (L)	None	501	0	ND
F22	Isoleucine zipper (L)	K483E + E488K	495	0	ND
F23	Isoleucine zipper (S)	None	495	0.3 ¹	stable
F46	Isoleucine zipper (S)	None	513	Did not express	ND
*Stability is defined as described in Example 7; ND: Not determined.					
¹ Expression level determined by Western Blot as described in Example 1.					

10 As can be seen in Table 1, the only constructs that were expressed were the Fibritin variant (F18) and F23. Although F18 was trimeric and showed expression, it was unstable upon storage at 4° C. In contrast, F23 was stable at 4° C, binds to the pre-fusion-specific

antibodies, but appeared to be monomeric. Therefore, both variants F18 and F23 were used to optimize for both stability and trimerization.

Next, several constructs were made in which the fusion peptide at the N-terminus of F1 was fixed by fusion to the C-terminus of the F2 domain. All constructs comprised a His-tag.

Several constructs were made including constructs in which both furin cleavage sites were mutated resulting in a soluble F protein that still contained the p27 peptide (i.e. F12, F15.1, and F17). In other constructs the 27 residue region (P27 loop) that is cleaved from the precursor F0 was replaced by an alternative closed loop or linking sequence: either by replacing the region of RSV-F by the 'homologous' region of PIV-5 F, the prefusion F protein that had been produced and crystallized successfully (F25), or by replacing the region by a minimal (GS)_n loop that would bridge the termini of F2 and F1 (F24), or by replacing the region by the central conserved region of RSV-G (F26). Homology modeling of RSV-F based on PIV-5 and *in silico* measurements resulted in the choice of a minimal loop of 5 amino acid residues between residues 108 and 136. As a linker, Gly (G) and Ser (S) residues were chosen which are flexible and polar and have a high chance to be accommodated (F24). Additionally, F137 was mutated to S because the local modifications caused by the loop could displace the hydrophobic F and cause instabilities. This is shown below. Also the R106 is mutated to Q and 27 residues (109-135) are replaced by GSGSG.

PAANN**RARREAPQYMNYTINTTKNLNVSISKRR**₁₃₆**FLGFLLGVG**

PAANN**QAR**₁₀₆**GSGSGR**₁₃₆**SLGFLLGVG**

As shown in Table 2, all variants showed no or very low expression except for the variant with the short GSGSG loop (F24) which showed a much higher expression (44 µg/ml) compared to wild type RSV F construct, i.e. a similar construct, without said linker

(F11). F24 which was trimeric, however, was unstable upon storage like all the other variants with a C-terminal Fibrin trimerization motif. All variants contained a HIS-tag.

5 **Table 2.** Expression and stability of RSV F constructs with different F1-F2 linkers

RSV Protein	Variant	Description					Stability*
		Trimerization motif	F1, F2 linker	Modifications	Termination point	Expr. (ug/ml)	
F11	B1	None	None	None	513	2.5	stable
F18	B1	Fibrin	None	None	513	2	unstable
F12	B1	Fibrin	p27	Furin site KO	513	0,1	unstable
F15.1	B1	None	p27	Furin site KO	525	0.5	ND
F17	A2	Fibrin	p27	Furin site KO	513	0	ND
F24	B1	Fibrin	Q_GSGSG_S	None	513	44	unstable
F25	B1	Fibrin	PIV	None	513	0	ND
F26	B1	Fibrin	G CR	None	513	0	ND

*Stability is defined as described in Example 7. Expression level determined as described in Example 1.

10 Next, the most favorable modifications were combined to find the optimal pre-fusion F polypeptides. Combinations were made of variants with the GSGSG loop, C-terminal truncation of F1, and the addition of either fibrin (SEQ ID NO: 4) or the Isoleucin Zipper (S) motif (SEQ ID NO: 3)(see Table 3).

Table 3. Expression and stability of RSV F constructs with combinations of optimizations according to Tables 1 and 2.

RSV Protein	Variant	Termination point	Description			Stability CR9501 epitope)	
			Trimerization motif	F1, F2 linker	(ug/ml)	Heat (°C)	Storage
F11	B1	513	None	None	2.5	48	Stable
F23	B1	495	Isoleucine zipper (S)	None	0.3	ND	Stable
F24	B1	513	Fibritin	Q__GSGSG_S	44	51	Unstable
F45	B1	495	Fibritin	None	0	ND	ND
F44	B1	495	Fibritin	Q__GSGSG_S	0	ND	ND
F49	B1	495	None	None	2	ND	Stable
F50	A2	495	None	None	2	ND	Stable
F43	B1	495	Isoleucine zipper (S)	Q__GSGSG_S	0.4	53	Stable
F47	A2	495	Isoleucine zipper (S)	Q__GSGSG_S	5	52	Stable
F56	B1	513	Isoleucine zipper (S)	Q__GSGSG_S	0,4	ND	Stable
F46	B1	513	Isoleucine zipper (S)	None	0	ND	unstable
F42	B1	513	None	Q__GSGSG_S	20	54	Stable
F57	A2	513	None	Q__GSGSG_S	2-10	54	Stable

ND is not determined

*Storage stability as determined in Example 7. *Heat stability as determined in Example 8.

5 Expression level as determined by Western blotting (described in Example 1)

Addition of the GSGSG-loop always increased the expression of functional constructs as well as the heat stability of the protein. Combination of the GSGSG-loop with the truncated F and isoleucine zipper (S) motif (F43, F47) showed good expression, heat stability and good stability upon storage at 4 °C. However, these variants were still monomeric. The isoleucine zipper (S) trimerization motif showed higher expression with a F variant that was C-terminally truncated F at position 495 (compare F43 with F56 and F23 with F46). In contrast, for variants with the Fibritin trimerization domain a truncation at position 513 showed high expression compared to truncation at position 495 which showed no expression (compare F24 with F44).

Because the HIS-tag could interfere with the native folding of the trimers, variants were made without the HIS-tag for the Fibritin and the isoleucine zipper (S) variant (Table 4).

Table 4. Expression and stability of RSV F constructs with and without HIS-tag

RSV Protein	Variant	Trimerization motif	F1, F2 linker	Termination point	Expression ug/ml	Trimerization %	Heat (°C)	Storage	Tags
F24	B1	Fibritin	Q_GSGSG_S	513	44	Trimeric (SEC)	51	unstable	His-tag
F24-	B1	Fibritin	Q_GSGSG_S	513	55	100% (Native)	ND	unstable	None
F47	A2	Isoleucine zipper (S)	Q_GSGSG_S	495	5	0% (Odyssey)	52	stable	His-tag
F47-	A2	Isoleucine zipper (S)	Q_GSGSG_S	495	10	2-5% (Odyssey)	53	stable	None
A2_F24	A2	Fibritin	Q_GSGSG_S	513	5,3	Trimeric (Native)	48,75	unstable	None

*Storage stability determined as described in Example 7; Heat stability determined as

described in Example 8; ND: not determined. ;

5

Strikingly, deletion of the HIS-tag increased expression in F47. Moreover, for F47 it increased the trimeric content slightly and for F24 it only increased the expression level moderately.

Next, several alternative trimerization domains and truncations were tested in

10 combination with the GSGSG-loop stabilized F variant (F47) (see Table 5). All variants have a GSGSG-loop and contain a HIS-tag.

Table 5. Expression and stability of RSV F variants with alternative trimerization domains

RSV Protein	Variant	Description				Trimerization %	Antibody binding	
		Trimerization motif	Modifications	Termination point	Expression (ug/ml)		CR9501	CR9503
F47	A2	Isoleucine zipper (S)	None	495	5	0%	+	+
P1	B1	Isoleucine zipper (S)	S502T	502	3.5	0%	+	+
Mat1	A2	Matrillin long	None	520	12	tri and hexamers	-	+
Mat2	A2	Matrillin short	None	516	0	ND	-	-
Mat3	A2	Matrillin short	None	495	1,5	ND	-	-
opt GCN	A2	GCN4II optimized	None	516	0	ND	-	-
opt GCN+L512K	A2	GCN4II optimized	L512K	516	1	ND	+	-

Antibody binding is defined as binding on the day of harvest (as described in Example 7;

+ indicates binding; - indicates no binding.

Expression level is determined as described in Example 1. ND: not determined

5

Only the matrillin 1 domain (Dames-SA et. al., Nat. Struc. Biol., 5(8), 1998) that contains both the N-terminal zipper domain and the C-terminal part with the cysteine residues that can potentially form inter trimeric disulfide bridges was found to enable higher expression levels than F47 (Table 5, Matrillin long). Moreover, the variant with the Matrillin long trimerization motif shows trimeric F proteins. However, the product did not bind to the pre-fusion specific Mab CR9501 and also showed hexameric species which makes the Matrillin 1 trimerization domain not suitable for production of trimeric native F protein. None of the matrillin-based or the GCN4II based zipper motifs showed increased expression or stability relative to F47 (Table 5, Matrillin short, GCN4II optimized). Again, truncation at 495 results in higher expression levels. Addition of a GCN4 motif which contained an optimized trigger sequence showed no expression.

GCN4 II is a trimerization domain that is used successfully for stabilizing the pre-fusion trimer of parainfluenza type 5 (Yin et al., Nature 439:38-44, 2006) and has also been tried by others to stabilize RSV pre-fusion F (as disclosed in e.g. WO2010/149743, WO2010/14975, WO2009/079796, WO2010/158613). The GCN4II trimerization domain

5 was evaluated and compared with the constructs that contain the Isoleucine Zipper (S) domain (SEQ ID NO: 3) or the Fibrin (SEQ ID NO: 4) domain (results shown in Table 6). These variants were also compared with another modifications, i.e. a short linker based on a single Lysine and the L512K mutation. All variants contained a HIS-tag.

10 **Table 6.** Expression and stability of RSV F variants with GCN4II, L512K and p27 replacement (single amino acid linker (K) between F1 and F2)

RSV Protein	Variant	Description					Stability	
		Trimerization motif	F1, F2 linker	Modifications	Termination point	Expr. (ug/ml)	Heat (°C)	Storage*
F18	B1	Fibrin	None	None	513	2	ND	unstable
F24	B1	Fibrin	Q_GSGSG_S	None	513	44	51	unstable
F43	B1	Isoleucine zipper (S)	Q_GSGSG_S	None	495	0,4	53	stable
P1	B1	Isoleucine zipper (S)	Q_GSGSG_S	S502T	502	3.5	54	ND
F42	B1	None	Q_GSGSG_S	None	513	16.1	54	stable
P2	B1	None	K	None	513	14,3	54	stable
P3	B1	GCN4II	None	L512K	516	0	ND	ND
P4	B1	GCN4II	K	L512K	516	0	ND	ND
P5	B1	GCN4II	K	L512K	516	0	ND	ND
P6	A2 †	GCN4II	K	L512K	516	0	ND	ND
P7	A2	GCN4II	K	L512K	516	0	ND	ND

Storage stability determined as described in Example 7; Expression levels determined as described in Example 1; Heat stability determined as described in Example 8; ND: not determined.

15 The short linkage between F1 and F2 appears to be comparable to the GSGSG loop. Addition of the GCN4II motif does not result in any F protein expression in any of the tested

constructs (i.e. the RSV A2 F sequence described in WO2010/149743 or WO2010/149745, the RSV A2 F sequence used according to the invention, nor the RSV B1 F sequence).

It was shown according to the invention that the introduction of these two types of modifications, i.e. introduction of a linking sequence and the heterologous trimerization

5 domain, was not enough to enable the expression of stable trimeric pre-fusion F protein.

Apart from the two main regions of instability that were stabilized, i.e. the HRB and the fusion peptide, as described above, other regions in the pre-fusion F protein also contribute and/or accommodate the dramatic refolding to post-fusion F, and more positions in the sequence can be optimized to stop the pre-fusion F protein from refolding. Therefore,

10 different amino acid residues in the HRA and HRB domain and in all domains that contact these regions in pre-fusion F were mutated to increase the pre-fusion structure stability, as described in the following Examples.

EXAMPLE 2

15 **Preparation of stable pre-fusion RSV F polypeptides – stabilizing mutations**

Because the trimeric content (for construct F47) and storage stability (for construct F24) was not optimal, further variants were made that contained point mutations to increase expression levels, stability and native trimeric structure. The results are shown in Table 7 and 8.

Table 7. Expression and stability of F47- variants

RSV Protein	Expression (ug/ml)	Trimerization %	Heat (°C)
F47-	10	2-5%	53
F47- + K465E	6	2.4%	ND
F47- + D479K	5	29%	50,77
F47- + K176M	13	5%	ND
F47- + K209Q	9	3%	52,9
F47- + S46G	38	11%	59,38
F47- + S215P	8	1-2%	57,21
F47- + N67I	15	2%	59,84
F47- + K465Q	18	2%	54,3
F47- S46G+N67I	31	6%	>60
F47- S46G+S215P	38	6%	>60
F47- K465Q+K209Q	12	1%	53,3
F47- K465Q+S46G	28	7%	57,7
F47- K465Q+N67I	17	2%	59
F47- K209Q+N67I	15	4%	>60
F47- K209Q+S215P	15	2%	56,7

ND: not determined; Expression level determined as described in Example 1. Heat stability determined as described in Example 8.

5 Nomenclature of mutations based on wt sequence (SEQ ID NO: 1).

All constructs are variants of F47- : type A2, Isoleucin Zipper (S) motif (SEQ ID NO: 3), GSGSG linker; termination point 495, no HIS-tag (SEQ ID NO: 16). As shown in Table 7,

many mutations increased the expression of F47-, but only the variant F47_S46G also showed a higher level of trimers besides the high expression.

Table 8 shows the results of the expression and stability of F24 variants. All variants were of

5 RSV type A2, with fibrin motif, GSGSG linker; termination point 513, no HIS-tag.

Table 8. Expression and stability of A2_F24- (SEQ ID NO: 19) variants

RSV Protein	Expression (ug/ml)	Storage	
		Endpoint	Association phase
A2_F24	5,3	69	ND
A2_F24 K508E	5,3	64	ND
A2_F24 K498E	1,7	ND	ND
A2_F24 E487I	25,0	10	ND
A2_F24 E487K	7,1	ND	ND
A2_F24 E487N	42,4	22	ND
A2_F24 E487P	12,8	46	ND
A2_F24 E487Q*	14,8	50	ND
A2_F24 E487R	8,7	59	ND
A2_F24 E487S	6,7	46	ND
A2_F24 E487Y	10,5	36	ND
A2_F24 D486N	31,2	19	ND
A2_F24 D479N	5,2	ND	ND
A2_F24 D479K	1,5	62	ND
A2_F24 E472Q	1,9	ND	ND
A2_F24 E472K	0,9	ND	ND
A2_F24 K465E	14,8	76	ND
A2_F24 K465Q*	13,6	92	Not stable

A2_F24 E463K	3,1	ND	ND
A2_F24 E463Q	6,0	ND	ND
A2_F24 G430S	4,8	ND	ND
A2_F24 N428R	5,2	35	ND
A2_F24 N426S	18,6	71	ND
A2_F24 K421N	9,2	75	ND
A2_F24 E328K	9,5	21	ND
A2_F24 T311S	3,5	70	ND
A2_F24 I309V	11,3	69	ND
A2_F24 D269V	0,0	ND	ND
A2_F24 S215P*	18,7	99	Stable
A2_F24 K209Q	31,4	63	ND
A2_F24 V207P	3,3	79	ND
A2_F24 I206P	5,4	55	ND
A2_F24 L204P	5,9	ND	ND
A2_F24 L203P	0,8	ND	ND
A2_F24 Q202P	4,4	ND	ND
A2_F24 K201Q	21,3	62	ND
A2_F24 D194P	1,9	ND	ND
A2_F24 L193P	6,5	42	ND
A2_F24 V192P	0,6	32	ND
A2_F24 V185N	50,2	38	ND
A2_F24 GV184EG	3,5	ND	ND
A2_F24 G184N	59,8	37	ND
A2_F24 V178P	14,8	23	ND
A2_F24 A177P	2,0	ND	ND
A2_F24 K176M	14,7	58	ND

A2_F24 K176E	0,7	ND	ND
A2_F24 N175P	34,3	55	ND
A2_F24 S169P	0,5	ND	ND
A2_F24 K168P	0,1	ND	ND
A2_F24 K166P	12,3	45	ND
A2_F24 V157P	0,2	ND	ND
A2_F24 E92D	47,4	94	Not stable
A2_F24 K85E	1,1	ND	ND
A2_F24 K80E	51,9	60	ND
A2_F24 K77E	22,4	ND	ND
A2_F24 N67I*	89,8	101	Stable
A2_F24 I57V		ND	ND
A2_F24 V156I	16,5	54	ND
A2_F24 S46G*	40,7	96	Not stable

The *marked constructs were tested for trimerization and were all found to be trimeric

Expression level determined as described in Example 1. Endpoint stability is shown here as the percentage of pre-fusion antibody binding (CR9501) after 5 days of storage at 4°C relative to day 1; Association phase stability is determined as described in Example 9.

5

Many mutations increased the expression of A2_F24-. For most mutations there was an apparent correlation between improved expression in F47- background (Table 7) and A2_F24- background (Table 8). N67I had more positive impact on F expression in A2_F24- background. The most significant increase in expression was obtained with the single point mutations: S46G, S215P, N67I, K80E, E92D, D486N, G184N, V185N, E487N, N175P, K209Q, E487I, E487Q, K77E, K201Q, N426S and K465Q. In the initial screening using the endpoint stability assay (Example 7) the variants with the highest expression showed the best stability upon storage as well (E92D, K465Q, K465E, N426S, S46G, S215P and N67I). To

10

evaluate if these mutations indeed were stabilizing the pre-fusion conformation, culture supernatants were diluted to 5 and 10 µg/ml based on quantitative western results and these were stored up to 33 days at 4°C. As single point mutants only N67I and S215P were completely stable over time (see Example 9).

- 5 Subsequently, several mutations that showed high expression and good stability of the pre-fusion conformation were combined to evaluate whether the stabilizations were additive or had a possible synergistic effect (Table 9).

Table 9. Expression and stability of variants of A2_F24 with two additional mutations.

RSV Protein	Expression (ug/ml)	stability*
A2_F24 K465Q + S46G	21,8	Not stable
A2_F24 K465Q + N67I	122,3	Stable
A2_F24 K465Q + E92D	10,5	Stable
A2_F24 K465Q + S215P	59,8	Stable
A2_F24 S46G + N67I	115,5	Stable
A2_F24 S46G + E92D	14,3	Not stable
A2_F24 N67I + E92D	134,2	Stable
A2_F24 N67I + S215P	152,1	Stable
A2_F24 E92D + S215P	49,1	Stable
A2_F24 K465Q+S215P	53,3	Stable
A2_F24 S46G+S215P	43,8	Stable

- 10 Storage stability refers to the association phase analysis illustrated in example 9.

Expression level was determined as described in Example 1.

All variants are variants of F24-: type A2, fibrin motif, GSGSG linker; termination point 513, binding to all Mabs, no HIS-tag (SEQ ID NO: 19).

When the previously identified point mutations were combined very interesting synergistic effects could be observed especially in terms of expression levels with the combinations involving N67I as the most potent. All produced double mutants where either N67I and S215P was included were stable after more than 30 days storage at 4 °C (Example 9). Strikingly, the mutation N67I was found to have the strongest effect on expression levels of pre-fusion F when included in the double mutants. Next, combinations with the S215P mutations resulted in a reasonable expression. Combination of N67I with S215P was selected since it led to a very high expression level, and because both point mutations were stable upon storage. Additionally it was observed that both N67I and S215P had the ability to stabilize some of the mutants that as single mutations were unstable indicating that the region where these two mutations are found is central for the conformation changes the protein undergoes during the transition to the post-fusion conformation.

According to the invention it thus has been shown that at least some mutations resulted in increased expression levels and increased stabilization of the pre-fusion RSV protein. It is expected that these phenomena are linked. The mutations described in this Example all resulted in increased production of pre-fusion F polypeptides. Only a selection of these polypeptides remained stable upon long storage (see Example 9). The stability assay that was used is based on the loss of the pre-fusion specific CR9501 epitope in the top of the pre-fusion F protein in a binding assay and it may not be sensitive enough to measure all contributions to stability of the whole protein. The mutation for which only increased expression is observed are therefore (very likely stabilizing) potential mutations that can be combined with other stabilizing mutations to obtain a pre-fusion F construct with high stability and high expression levels.

Next, it was verified whether the N67I - S215P double mutation, like the single mutations, was able to stabilize point mutations that as single mutants were deemed unstable based on the criteria used. Extra mutations were selected based on the favorable expression levels and stability according to Table 8. Triple mutant RSV-F variants were constructed and
5 tested for expression levels and stability (Table 10).

Table 10. Expression and stability of variants of F24_N67I +S215P with one additional mutation.

RSV Protein	Expression (ug/ml)	stability*
A2_F24 N67I + S215P+K507E	344,6	++
A2_F24 N67I + S215P+E487I	239,4	+++
A2_F24 N67I + S215P+E487N	285,2	+++
A2_F24 N67I + S215P+E487Q	360,7	+++
A2_F24 N67I + S215P+E487R	130,9	+++
A2_F24 N67I + S215P+D486N	292,6	+++
A2_F24 N67I + S215P+D479N	97,1	+++
A2_F24 N67I + S215P+K465Q	283,3	+++
A2_F24 N67I + S215P+N426S	316,3	+++
A2_F24 N67I + S215P+K421N	288,4	+++
A2_F24 N67I + S215P+K209Q	245,0	+++
A2_F24 N67I + S215P+K201Q	231,9	+++
A2_F24 N67I + S215P+V185N	445,1	+++
A2_F24 N67I + S215P+G184N	326,7	+++
A2_F24 N67I + S215P+E92D	308,8	+
A2_F24 N67I + S215P+K80E	210,6	+
A2_F24 N67I + S215P+S46G	199,4	+++

10 All variants are variants of A2_F24_N67I +S215P type A2, fibritin motif, GSGSG linker; termination point 513, binding to all Mabs, no HIS-tag (SEQ ID NO: 21).

*stability refers to the association phase analysis illustrated in example 9.

+ means <10% loss of CR9501 binding after 5 days; ++ means <5% loss of CR9501 binding after 5 days; +++ means 0% loss of CR9501 binding after 5 days.

Again, an additive effect on the expression levels was observed. As expected D479N and E487R triple mutants express at somewhat lower levels because the single mutants were also among the lowest of the selected mutations (Table 8). Because of the stabilizing effect of the N67I+S215P mutation, additional mutations that are unstable as single mutants, resulted in stable pre-fusion F variants when they were added to the A2_F24 N67I+S215P background. Some very illustrative examples are the triple mutants with the additional V185N, G184N or E487N which showed high expression but low stability as single mutants (Table 8) but showed even higher expression and were highly stable when added to the A2_F24 N67I+S215P background.

Stabilizing mutations also stabilize RSV-F protein from other strains and also in processed F variant.

Several mutations that showed high expression and good stability of the pre-fusion conformation were applied to RSV F proteins of other strains (SEQ ID NOs 69 and 70) and were applied to a RSV A2 F variant without furin cleavage site mutations (F18: SEQ ID NO 71) to evaluate whether the modifications are a universal solution to stabilize RSV prefusion F.(Table 11)

Table 11. Expression and stability of variants of A2_F18 with additional mutations and F from strain B1 (SEQ ID NO: 2) and type A CL57-v224 (SEQ ID NO: 69).

RSV protein	Seq ID	Relative* expression (CR9503)	Stability** after day 5, %
A2_F18	71	0.018	0.0
A2_F18 N67I		0.449	73.2
A2_F18 S215P		0.129	9.1
A2_F18 E487Q		0.006	NA
A2_F18 N67I, S215P	72	0.484	103.4
A2_F18 N67I, E487Q		0.340	92.1
A2_F18 N67I, S215P, E487Q	76	0.355	92.7
A2_F18 N67I, S215P, E92D	78	0.318	96.0
A2_F18 N67I, S215P, D486N	79	0.522	101.3
A2_F18 N67I, S215P, K201N	77	0.643	102.7
A2_F18 N67I, S215P, K66E		0.800	103.0
A2_F18 N67I, S215P, S46G, K66E		0.820	103.5
A2_F18 N67I, S215P, E487Q, K66E		0.704	99.5
A2_F18 N67I, S215P, E92D, K66E		0.905	98.8
A2_F18 N67I, S215P, D486N, K66E		0.863	96.6
A2_F18 N67I, S215P, K201N, K66E		1.021	105.5
A2_F18 N67I, S215P, D486N, K66E, I76V		0.594	95.0
B1_N67I, S215P	73	0.434	90.9
B1_N67I, S215P loop	22	0.552	108.2
CL57v224_N67I, S215P	74	0.698	94.9
CL57v224_N67I, S215P loop	75	0.615	98.4

5 *Protein expression (concentration in the supernatant of transiently transfected cells) was measured by Quantitative Octet method.*

** Relative expression is normalized to expression of A2_F24_N67I, S215P, E487Q (seq ID #33)*

*** Stability - is expressed as % protein concentration measured after storage at 4C for 5 days, relatively to the day of harvest. The concentrations were measured by Quantitative Octet method*

10 *using CR9501 antibody. NA - data not available: no CR9501 binding was detected.*

When the previously identified point mutations were introduced in A2_F18 (SEQ ID No. 71), the stability and expression levels were very similar compared with the single chain

F24 (SEQ ID No. 21) variant that contained a short loop between F1 and F2. Again, synergism was observed showing higher expression and stability when mutations were added to variants that contained the N67I or the double mutation N67I, S215P. The double point mutation N67I, S215P did not only stabilize the pre-fusion F of the A2 strain but also pre-fusion of of B1 and CL57-v224 strain (Table 11).

Stabilizing mutations also stabilize full length RSV-F protein.

Several mutations that showed high expression and good stability of the pre-fusion conformation in the soluble version of RSV-F corresponding to the ectodomain, were applied to the full length RSV-F protein. The mutations were introduced in full length RSV-F with or without furin cleavage site mutations. No trimerization domain was fused to these variants (Table 12)

Table 12. Expression and stability of variants of full length versions of A2_F18 and A2_F24 with additional mutations.

Amino acid substitutions	RSV F protein variant*		Attributes	
	SEQ ID No	F1, F2 linker	Expression, fold increase**	Heat-stability***
None (F A2 wildtype, full length)	1	none	1	-
N67I		none	1.4	N.D.
S215P		none	1.4	N.D.
E92D		none	1.4	N.D.
N67I, K465Q		none	1.4	N.D.
N67I, S46G		none	0.2	N.D.
N67I, E92D		none	1.4	N.D.
N67I, K80E		none	2.3	N.D.
N67I, G184N		none	1.5	N.D.
N67I, V185N		none	1.4	N.D.
N67I, E487Q		none	2.5	N.D.
N67I, S215P, V185N		none	2.7	N.D.
N67I, S215P, K508E		none	3.0	N.D.
N67I, S215P, K80E		none	3.1	N.D.
N67I, S215P, K465Q		none	2.9	N.D.
N67I, S215P	80	none	2.4	++

N67I, S215P, G184N		none	7.6	++
N67I, S215P, E92D	82	none	6.8	N.D.
N67I, S215P, S46G	88	none	6.8	+
N67I, S215P, D486N	86	none	5.9	+++
N67I, S215P, E487Q	84	none	6.2	N.D.
N67I, S215P, S46G, K66E		none	12.1	+++
N67I, S215P, D486N, K66E		none	9.2	+++
N67I, S215P, S46G, E92D, K66E		none	11.8	+++
N67I, S215P, S46G, E487Q, K66E		none	11.0	+++
N67I, S215P, S46G, D486N, K66E		none	10.5	+++
N67I, S215P, D486N, K66E, I76V		none	7.2	+++
N67I, S215P, S46G, K66E, I76V		none	9.7	+++
N67I, S215P, S46G, K80E		none	4.5	N.D.
N67I+S215P+G184N+K80E+E92D+E487Q+S46G		none	9.1	N.D.
None		Q_GSGSG_S	3.8	-
N67I, S215P	81	Q_GSGSG_S	6.2	N.D.
N67I, S215P, G184N		Q_GSGSG_S	7.2	++
N67I, S215P, E92D	83	Q_GSGSG_S	5.9	N.D.
N67I, S215P, S46G	89	Q_GSGSG_S	5.3	++
N67I, S215P, D486N	87	Q_GSGSG_S	5.2	+++
N67I, S215P, E487Q	85	Q_GSGSG_S	4.6	N.D.
N67I, S215P, S46G, K66E		Q_GSGSG_S	11.7	+++
N67I, S215P, D486N, K66E		Q_GSGSG_S	13.8	+++
N67I, S215P, D486N, K66E, I76V		Q_GSGSG_S	6.8	+++
N67I+S215P+G184N+K80E+E92D+E487Q+S46G		Q_GSGSG_S	3.6	N.D.

Expression level determined using FACS. N.D. – not determined. *all variants are based on RSV A2 F protein sequence. ** comparing to wild type protein, fold increase of MFI on 9503.

Stability was assessed by heat treatment of the HEK293T cells for 5 - 10 minutes at 46, 55.3, 60 °C.

*** legend for the stability readout

- 5 - decrease in binding to prefusion – specific Mab CR9501 binding after 46 °C (e.g. wild type)
 + slight decrease of CR9501 binding after 46 °C but not to same strong extent as wild type
 ++ no change in CR9501 binding up to 60 °C, at 60 °C some decrease in CR9501 binding
 +++ no change in CR9501 binding at 60 °C

- 10 The previously identified stabilizing point mutations were also stabilizing in the full length F protein. The increase in expression level was less pronounced but showed the same trend. This may be caused by the different background the mutations were introduced in but

may also be caused by the different quantification method (FACS versus Western blot) and a biological maximum of expression due to recycling of surface proteins. Introduction of the linking sequence (or short loop) increased expression and stability and the point mutations did so too. The point mutations were not or hardly synergistic with the short loop (similar as

5 to what we found for the soluble protein (Table 9-11)

Because the point mutation at position 67 had such positive effect on expression level and stability, all amino acid substitutions were tested for this position to study whether the most optimal were chosen or whether these positions can be improved. (Table 13)

10

Table 13. Full substitution analysis of expression and stability for position 67 in the A2_F24 background.

Amino acid substitution	Relative Expression*	Stability** after day 4, %	Stability** after day 10, %
N67A	1.696	0.0	0.0
N67C	1.759	16.7	0.0
N67D	1.702	0.0	0.0
N67E	1.357	0.0	0.0
N67F	2.565	102.2	108.1
N67G	0.853	NA	NA
N67H	1.509	0.0	0.0
N67I	3.773	98.2	102.7
N67K	0.487	NA	NA
N67L	3.609	107.5	96.4
N67M	2.579	87.3	78.7
N67P	2.414	14.3	0.0
N67Q	0.955	NA	NA
N67R	0.523	NA	NA
N67S	1.277	0.0	0.0
N67T	1.577	0.0	0.0
N67V	2.457	84.2	77.0
N67W	1.794	99.9	104.3
N67Y	1.830	61.3	45.8

* Relative expression - protein concentration was measured by Quantitative Octet method using

15 CR9503 antibody and expressed relative to concentration of A2_F24 (SEQ ID #19)

** Stability - is expressed as % protein concentration measured after storage at 4C for 5 and 10 days, relatively to the day of harvest. The concentrations were measured by Quantitative Octet method using CR9501 antibody. NA - data not available: no CR9501 binding was detected.

As shown in Table 13, primarily hydrophobic residues and particularly Ile, Leu and Met at position 67 were able to increase expression and stability. Ile is the residue that increased expression and stability most. Residues Glu and Gln, the smallest residue Gly and the positively charged residues Arg and Lys had the most destabilizing effect at position 67 on the prefusion conformation.

According to the invention, the amino acid mutations that stabilize the pre-fusion conformation of the RSV F protein can be grouped into different categories that stabilize the conformation in different manners. The strategies for pre-fusion F stabilization are based on the homology model of RSV-F that was based on the PIV5 crystal structure (Yin et. al., 2006) and the alignment on page 27.

Amino acid residues 67 and 215:

The amino acid residues at positions 67 and 215 are very close in the 3D structure of both the pre-fusion model and post-fusion crystal structure. The residues are close to the conserved disulfide bridge in the top of the DIII region that forms the hinge along which the HRA region refolds into the long elongated coiled coil extended helical trimer. Mutations in this region will influence the hinge function and therefore the mutations that were introduced stabilize the pre-fusion conformation by obstruction of the hinge function.

Amino acid residues 77, 80

The amino acid residues at positions 77 and 80 are located within the long helix (residues 76-98) at the C-terminus of F2 that is in close contact to the ensemble of secondary structures in DIII at the N-terminus of F1 that refold into the long coiled coil structure of the post-fusion conformation. Since these two regions have to be separated during the refolding from pre- to

post-fusion, amino acids in this region that prevent this separation would stabilize the pre-fusion conformation. Because these two regions should part during refolding, some of the residues can be optimized to strengthen the interaction. An example of a repulsion that was observed was between the positively charged Lys80. Mutation of Lys80 to the negatively charged Glu residue increased the expression of pre-fusion F. Due to the sequential transition to the post-fusion conformation these mutations can be combined with other stabilizing mutations like N67I and S215P to get the full benefit of this stabilization, as shown in Table 10.

10 Amino acid residue 92

The amino acid residue at position 92 is also located within the long helix (residues 76-98) at the C-terminus of F2 that is in close contact to the ensemble of secondary structures in DIII at the N-terminus of F1 that refold into the long coiled coil structure of the post-fusion conformation. When this helix is separated from the HRA region, it is pulled to the DIII region that contains the Synagis epitope (epitope II) (Arbiza et al., J. Gen. Virol. 73:2225–2234, 1992) and the negatively charged Glu92 moves very close to the positively charged Arg282 in the postfusion conformation. Mutations that reduce this pull will stabilize the pre-fusion conformation. Mutation of Glu92 to a conserved Asp residue will reduce the pull because it is not able to reach Arg282.

20

Amino acid residues 486, 487

The amino acid residues 486, 487 and 489 at the top of HRB in the pre-fusion conformation form a negatively charged patch Mutation of Glu487 to Asn or Ile increased pre-fusion F expression. Mutations of Asp486 into Asn or Gln and/or Glu489 into Asn, Ile or Gln will have the same effect.. Due to the sequential transition to post-fusion these mutations can be

25

combined with other stabilizing mutations like N67I and S215P to get the full benefit of this stabilization, as shown in Table 10 for e.g. D486N.

Amino acid residues 175, 184, 185

- 5 In order to refold from the pre-fusion to the post-fusion conformation, the region between residue 175 and 193 has to transform from a loop – beta hairpin to a helix.

This region demonstrates the most dramatic structural transition. Part of this region actually has the highest alpha-helix prediction. The actual helical structures in the pre-fusion model are shown below in grey highlights. This whole region is transformed into one large helix

- 10 when it refolds to the post-fusion conformation. In the bottom sequence the residues are highlighted in grey with the highest helix prediction based on Agadir (<http://agadir.crg.es/>). It is clear from this comparison that the C-terminal part that is maintained in a beta-hairpin in the pre-fusion conformation, (residues 187-202) has a high tendency to form a alpha-helix.

15 150 160 170 180 190 200 210

SGVAVSKVLHLEGEVNIKISALLSTNKAVVSLNGVSVLTISKVLDLKNYIDKQLLPPIVKNKQSC

hhhhhh hhhhhhh hhhhh sssssss sssssss hhhhhhhh

SGVAVSKVLHLEGEVNIKISALLSTNKAVVSLNGVSVLTISKVLDLKNYIDKQLLPPIVKNKQSC

20

The sequence of residues 150 – 212 of RSV-F is shown above. On the second line the secondary structures of the top line are indicated by h (for helix) and s (for strands) based on the PIV-5 3D homology model. Helices are highlighted with grey shading. The bottom line is the same sequence in which the helices are shades grey based on the helix propensity of the

- 25 sequence

Therefore, a Proline was introduced at position 175 to stabilize this turn and to prevent refolding into a helix which as a single mutation increased the expression level, indicating that it stabilizes the pre-fusion conformation and enables better processing of the protein. For the turn in the hairpin (residues 184, 185) the Brookhaven database was searched for a

5 structurally homologous hairpin from a stable protein that does not refold. A high structural homology was discovered with a hairpin loop in Protein Kinase A (pdb code 3FHI). According to the alignment shown below, residues 184 Gly or 185Val were replaced by Asn in order to stabilize this turn and prevent it from refolding.

VVSLSN**GV**SVLTSKV HRAb1b2 178-192

10 EMDVYV**NN**EWATSVG 3fhi:B 179-193

These mutations can be combined with other stabilizing mutations like N67I and S215P to get the full benefit of this stabilization as shown in Table 10.

15 Amino acid residues 421, 426 and 46

The amino acid residues at positions 421 and 426 are in a loop in the DII region. Residue S46 is on a strand that crosses from DI to DIII. The amino acid residue at position 426 was mutated into serine and the amino acid residue at position 46 was mutated into glycine. These mutations increased stability and pre-fusion expression levels.

20

Amino acid residue 465

The amino acid residue Lys465 is located in another region that goes through a large conformational change. Lys465 is located on a cross-over loop that connects the top of the DII region to HRB. Because the HRB region moves up from the bottom to the top and

25 complexes with HRA to make the six helix bundle, the cross-over loop also relocates from

bottom to top. This loop must therefore be metastable in order to allow detachment of DII and reposition in another environment. Lys465 on the cross-over loop is close to Lys445 on the DII region. Mutation of Lys465 to either Gln or Glu neutralizes the repulsion and increased stability and pre-fusion F expression levels.

5

EXAMPLE 3

Expression of pre-fusion F protein

Expression plasmids encoding the recombinant pre-fusion RSV F protein were generated by standard methods widely known within the art, involving site-directed
10 mutagenesis and PCR. The expression platform used was the 293Freestyle cells (Life Technologies, Renfresshire, UK). The cells were transiently transfected using 293Fectin (Life Technologies) according to the manufacturer's instructions and cultured in a shaking incubator for 5 days at 37°C and 10% CO₂. The culture supernatant was harvested and spun for 5 min at 300 g to remove cells and cellular debris. The spun supernatant was subsequently
15 sterile filtered using a 0.22 um vacuum filter and stored at 4°C until use.

EXAMPLE 4

Purification of pre-fusion RSV F protein

The recombinant polypeptides were purified by a 2-step purification protocol
20 applying a cat-ion exchange column for the initial purification and subsequently a superdex200 column for the polishing step to remove residual contaminants. For the initial ion-exchange step the culture supernatant was diluted with 2 volumes of 50 mM NaOAc pH 5.0 and passed over a 5 ml HiTrap Capto S column at 5 ml per minute. Subsequently the column was washed with 10 column volumes (CV) of 20 mM NaOAc, 50mM NaCl, 0.01%
25 (v/v) tween20, pH 5 and eluted 2 CV of 20 mM NaOAc, 1M NaCl, 0.01% (v/v) tween20, pH

5. The eluate was concentrated using a spin concentrator and the protein was further purified using a superdex200 column using 40mM Tris, 500mM NaCl, 0.01% (v/v) tween20, pH 7.4 as running buffer. In Figure 1A the chromatogram from the gel filtration column is shown and the dominant peak contains the pre-fusion RSV F protein. The fractions containing this peak were again pooled and the protein concentration was determined using OD280 and stored at 4°C until use. In Figure 1B a reduced SDS-PAGE analysis of the final protein preparation is shown and as can be seen the purity was >95%. The identity of the band was verified using western blotting and protein F specific antibodies (not shown).

10 EXAMPLE 5

NativePAGE

For initial determination of the multimeric state of the pre-fusion F polypeptides according to the invention, culture supernatants from transiently transfected cells were analyzed in a NativePAGE Bis-Tris gel system (Life Technologies). Subsequently the gels were blotted using the iBlot technology according to the manufacturer's instructions (Life Technologies). An RSV F protein specific antibody CR9503 (sequences given below in Table 17) was used as primary probe for the detection of pre-fusion RSV F protein and followed by a HRP conjugated mouse anti-human IgG (Jackson ImmunoResearch, West Grove, PA) or a IRDye800CW conjugated affinity purified anti-human IgG (rabbit) (Rockland Immunochemicals, Gilbertsville, PA). The blots were developed with either standard film (Codak) or using the Odyssey CLx infrared imaging system. Figure 2 shows the NativePAGE analysis of supernatants from monomeric F47-, (lane 1), post-fusion and primarily trimeric RSV F protein (lane 2) and purified pre-fusion RSV F protein (lane 3), showing that after purification only trimeric species are present in the pre-fusion RSV F protein preparation

since it migrates similarly to the post-fusion trimer band. This is supported by the elution volume from the gel filtration column as well (Figure 1A).

EXAMPLE 6

5 **Quantitative Western blotting**

For quantification of the pre-fusion RSV F protein constructs, quantitative Western blotting was used. Dilutions of culture supernatants were run reduced on 4-12% (w/v) Bis-Tris NuPAGE gels (Life Technology) and blotted using the iBlot technology (Life Technology). The blots were probed with CR9503 (as described above) and developed with
10 either a conjugated mouse anti-human IgG (Jackson ImmunoResearch, West Grove, PA) or a IRDye800CW conjugated affinity purified anti-human IgG (rabbit) (Rockland Immunochemicals, Gilbertsville, PA). The protein quantities were then estimated using a dilution serie of purified RSV standard protein and either the Odyssey CLx infrared imaging system or by eye. In Figure 3 the effects relative to the A2_F24 (SEQ ID NO: 19) construct in
15 terms of overall expression levels can be seen. It was shown that single mutations increased the expression level up to 5-fold. If double mutants of some of these mutations were generated synergistic effects could be observed and in some cases a further increased expression up to 11-fold over the A2_F24 was observed.

20 EXAMPLE 7

Endpoint stability assay

The verification of the pre-fusion conformation of the expressed polypeptides according to the invention was done using the BioLayer Interferometry (Octet) technology using the pre-fusion specific antibodies CR9501 or CR9502, or the non-conformation specific
25 antibody CR9503, which comprises the heavy and light chain variable regions of

Motavizumab. The antibodies were biotinylated by standard protocols and immobilized on Streptavidin biosensors (ForteBio, Portsmouth, UK). The procedure was as follows. After equilibration of the sensors in kinetic buffer (ForteBio) for 60s the Tipss were transferred to PBS with 5 ug/ml of the desired antibody. The loading was carried out for 250s.

- 5 Subsequently another equilibration step was included for 200s in kinetic buffer. Lastly the tipss were transferred to the expression culture supernatant containing the pre-fusion RSV F polypeptides and binding response (nm) after 1200s was recorded. This phase is also referred to as the association phase. This was done immediately after harvest (day 1) as well as 5 days later (day 5) and the difference in the CR9501 binding was used as a screening tool to
- 10 identify mutations capable of stabilizing the pre-fusion conformation. A construct was deemed stable if less than 20% loss of binding was observed at day 5 and if more than 20% loss of binding was observed it was deemed unstable. Stable constructs could then undergo a more stringent stability test if needed. The data analysis was done using the ForteBio Data Analysis 6.4 software (ForteBio).

15

EXAMPLE 8

Heat stability assay

- The stabilizing potential of introduced features into the RSV F polypeptides was estimated by heat stress. For that purpose culture supernatant from transiently transfected
- 20 cells or purified protein was heated using a range of temperatures. The samples were subsequently cooled on ice to prevent further heat induced conformational changes and probed using the CR9501 antibody on the octet technology platform as described in Example 7. The responses obtained at end of the association phase at the different temperatures were plotted as a function of the temperature and fitted by non-linear regression using the Prism
- 25 software. This resulted in an estimation of the temperature where the antibody binding level

is 50% of the maximum and this value could be used to compare different constructs in terms of pre-fusion heat stability. In Figure 4 the unmodified ectodomain (SEQ ID NO: 13) and the A2_F24 N67I+S215P construct (SEQ ID NO: 21) are compared. It can be observed that temperature-induced stress has a less effect on the A2_F24 N67I+S215P construct (SEQ ID NO: 21) as compared to the unmodified ectodomain. Therefore it can be concluded that the stabilizing motifs introduced in the polypeptides according to the invention, i.e. the trimerization site, the F1-F2-linker and the 2 point mutations lead to a more stable pre-fusion F protein.

10 EXAMPLE 9

Association phase stability assay

To assess the stability of various point mutations the octet binding assay a variation of the previously described endpoint stability assay (Example 7) was developed. The association phase analysis was implemented due to the very high expression levels of some point mutants since it is more stringent and completely prevents expression level bias. The CR9501 antibody was also used but instead of selecting the binding response at the end of the association phase the entire association curve was used, to reduce potential concentration bias of the endpoint assay. This was done using the data points from the entire association phase of the experiment using the indicated A2_F24 point mutants. The data were compensated for the amount of bound antibody on the chip. The measurements were done at days 1, 5 and 33, and the shapes of the curves from the three days were compared. If identical curves were obtained the construct was deemed stable and if not, unstable. In Figure 5, the analysis of four different variants can be seen. Unstable protein pre-fusion constructs can be identified by a time-dependent loss of CR9501 binding (A2_F24, K465Q, S46G), while stable pre-fusion constructs (N67I) displayed no such decrease. The mutation E92D appeared

to fall into a group in between the two having an intermediate stability since only minor changes in the shape of the curve were observed. In Figure 6 selected point mutations have been combined to make double mutants and these were analyzed. As can be seen, the different mutations displayed different phenotypes in terms of stability and stability

5 induction. When the polypeptides comprise the mutations K465Q or S46G alone or in combination, all three, i.e. the two single and the double mutants, are unstable and pre-fusion-specific antibody binding is lost over time. When the mutation S46G is combined with E92D, which previously was shown to have an intermediate stability as a single mutation, no change in stability could be observed, indicating that the E92D mutation cannot correct unstable
10 protein constructs. When the mutation N67I was combined with either the S46G or E92D mutation the result is a completely stable construct. This could also be observed when the S215P mutation was combined with the E92D mutation showing the unique potential of these two mutations to stabilize unstable pre-fusion constructs.

15 EXAMPLE 10

Quantitative Octet

To measure concentration of the pre-fusion RSV F protein in cell culture supernatants, quantitative Octet-based method was used. The CR9501 and CR9503 antibodies were biotinylated by standard protocols and immobilized on Streptavidin
20 biosensors (ForteBio, Portsmouth, UK). Afterwards, the coated biosensors were blocked in mock cell culture supernatant. Quantitative experiment was performed as follows: temperature 30C, shaking speed 1000 rpm, time of the assay 300 sec. Concentration of the protein in the cell culture supernatant was calculated using standard curve. The standard curve was prepared for each coated antibody using the A2_F24_N67I+S215P (SEQ ID# 21)
25 protein, diluted in mock cell culture supernatant. The measurement was done on the day of

supernatant harvest (day1) and after storage of the supernatant at 4C for 5 days or longer. The difference in the concentration determined with the CR9501 was used as a screening tool to identify mutations capable of stabilizing the pre-fusion conformation. A construct was deemed stable if less than 20% decrease of measured concentration was observed at day

5 5.The data analysis was done using the ForteBio Data Analysis 6.4 software (ForteBio).

EXAMPLE 11

FACS analysis and heat stability

Expression plasmids encoding the recombinant full length RSV F protein were
10 generated by standard methods widely known within the art, involving site-directed
mutagenesis and PCR. The HEK293-T cells were transiently transfected using 293Fectin
(Life Technologies) according to the manufacturer's instructions and cultured for 48 hours at
37°C and 10% CO₂. The cell were detached from cell culture dishes using FACS buffer (5
mM EDTA, 1% FBS in PBS), washed and resuspended in the same buffer. Cells were stained
15 for the surface RSV F protein by biotinylated CR9501 or CR9503 antibodies, followed by
APC-labeled streptavidin. For discrimination between live and dead cells Propidium iodide
was added to the cell suspension at the end of the staining procedure. The cells were analyzed
on FACS Canto (BD Biosciences) according to standard methods well known to any person
skilled in the art. The data analysis was done using the FlowJo 9.0 software (Tree Star Inc.).
20 Mean fluorescence intensity (MFI) was calculated for the population of live APC-positive
cells.

The stabilizing potential of introduced features into the full length membrane-bound
RSV F was estimated by heat stress. The cells 48 hours after transfection were detached from
cell culture dishes as described above and cell suspension was heated for 5-10 minutes using
25 a range of temperatures (37, 46, 55.3, 60°C). The cells were subsequently stained and

analyzed by FASC as described above. MFI was calculated for the population of live APC-positive cells. Percent of APC-positive cells was calculated for the live cell population.

Staining with the CR9503 resulted in similar MFI and %APC-positive cells in samples subjected to heat shock with increasing temperatures. Staining with CR9501 was decreasing
 5 in cell samples transfected with unstable proteins. Loss of the CR9501 binding indicated loss of the pre-fusion RSV F protein on the cell surface.

EXAMPLE 12

Preclinical evaluation of prefusion F immunogenicity

10 To evaluate the immunogenicity of a stabilized pre-fusion RSV F (A2F24,N67I, S215P) (SEQ ID NO: 21) we immunized mice according to Table 14 with 0.5 or 5 ug in a prime – boost regimen at week 0 and week 4. As shown in Figure 7, mice immunized with pre-fusion F showed higher VNA titers than mice immunized with post-fusion RSV F.

15 **Table 14.** Immunization scheme

Group	Preparation	Dose	Adjuvant	N
1	Post-fusion F	0.5 µg	-	9
2	Post-fusion F	5 µg	-	9
3	Pre-fusion F	0.5 µg	-	9
4	Pre-fusion F	5 µg	-	9
5	Post-fusion F	0.5 µg	Poly(I:C)	9
6	Pre-fusion F	0.5 µg	Poly(I:C)	9
8	FI-RSV	1/75	-	8
9	PBS		-	3

Next, cotton rats were immunized with two different doses of RSV-F in either the post-fusion or the pre-fusion conformation (Table 15). Animals were immunized i.m. at week 0 and week 4. Figure 8 demonstrates high neutralizing antibody titers at the day of challenge (week7).

5

Table 15. Groups, immunogen and dose for immunogenicity evaluation and efficacy in cotton rats

Group	Preparation	Dose	Adjuvant
1	Post-fusion F	0.5 ug	-
2	Post-fusion F	5 ug	-
3	Pre-fusion F	0.5 ug	-
4	Pre-fusion F	5 ug	-
9	Pre-fusion F	0.5 ug	Poly IC
10	Pre-fusion F	5 ug	Poly IC
11	Pre-fusion F	0.5 ug	Adju Phos
12	Pre-fusion F	5 ug	Adju Phos
13	Ad26.RSV.F _{A2}	10 ⁸	-
14	PBS	-	-

Five days after challenge the lung and nose viral load was measured (see Figure 9).

- 10 As shown, the pre-fusion F polypeptides according to the invention are able to induce a strong protective immune response that reduced viral load in the lung and even in the nose.

Table 16. 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 17. Antibody sequences

Ab	VH domain	VH CDR1	VH CDR2	VH CDR3
CR9501	Amino acids 1-125 of SEQ ID NO: 53	GASINSDNYYWT (SEQ ID NO:54)	HISYTGNTYYTPSLKS (SEQ ID NO:55)	CGAYVLISNCGWFDS (SEQ ID NO:56)
CR9502	Amino acids 1-121 of SEQ ID NO:57	GFTFSGHTIA (SEQ ID NO:58)	WVSTNNGNTEYAQKI QG (SEQ ID NO:59)	EWLVMGGFAFDH (SEQ ID NO:60)

Ab	VL domain	VL CDR1	VL CDR2	VL CDR3
CR9501	Amino acids 1-107 of SEQ ID NO: 61	QASQDISTYLN (SEQ ID NO: 62)	GASNLET (SEQ ID NO:63)	QQYQYLPYT (SEQ ID NO:64)
CR9502	Amino acids 1-110 of SEQ ID NO: 65	GANNIGSQNVH (SEQ ID NO:66)	DDRDRPS (SEQ ID NO:67)	QVWDSSRDQAVI (SEQ ID NO:68)

The amino acid sequence of several of the pre-fusion RSV F constructs is given

- 5 below. It is noted that the amino acid numbering in the different constructs described herein is based on the wild-type sequence (SEQ ID NO: 1), which means that all amino acids from position 1 to and including position 108 of the pre-fusion constructs correspond to the amino acid positions 1-108 of the wild-type sequence, whereas the numbering of the amino acids from position 138 to the end is shifted 22 amino acids, i.e. L138 in the wild-type sequence
- 10 (SEQ ID NO: 1) corresponds to L116 in all the pre-fusion constructs. This is due to the fact that a deletion has been made in the pre-fusion constructs i.e. the insertion of the GSGSG linker the actual numbering in F1 is not the same between constructs. Thus, the numbering used with respect to the specific mutations according to the invention, e.g. S215P, refers to the position of the amino acid in the wild type sequence.

Sequences**RSV F protein A2 full length sequence (SEQ ID NO: 1)**

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
5 LSNIKKNKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMN
YTLNNAKKTNTVTLSSKKRKRRLGFLGVGSAIASGVAVSKVLHLEGEVNIKSALLS
TNKAVVSLSNGVSVLTSTKVLDLKNIYIDKQLLPIVNVKQSCSISNIETVIEFQQKNNRLL
ITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSI
IKEEVLAYVYVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
10 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDFNPKYDCKIMTSKTDVSSSV
ITSLGAIVSCYGKTKCTASNKNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVNVKQE
GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
TNIMITTHIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

15

RSV F protein B1 full length sequence (SEQ ID NO: 2)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCNGTDTKVLIKQELDKYKNAVTELQLLMQNTPAANNRARRREAPQYMN
YTINTTKNLNVSISKRRKRRLGFLGVGSAIASGIAVSKVLHLEGEVNIKNALLSTN
20 KAVVSLSNGVSVLTSTKVLDLKNIYINNQLLPIVNVQSCRISNIETVIEFQQKNSRLLEIN
REFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIK
EEVLAYVYVQLPIYGVITPCWKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSF
FPQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSL
GAIVSCYGKTKCTASNKNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVNVKLEGKN
25 LYVKGEPIINYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELLHNVNTGKSTTNI
MITTHIIVILLSLIAIGLLLYCKAKNTPVTLSKDQLSGINNIAFSK

SEQ ID NO: 3

EKKIEAIEKKIEAIEKKIEA

30

SEQ ID NO: 4

GYIPEAPRDGQAYVRKDGWVLLSTFL

SEQ ID NO: 5

GSGSG

F8: RSV A2, wt ectodomain (SEQ ID NO: 13)

5 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKNKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMN
 YTLNNAKKTNTVLSKKRKRRLGFLGFGVGSASGVAVSKVLHLEGEVNNIKSALLS
 TNKAVVSLSNGVSVLTSTKVLDLKNYIDKQLLPVNNKQSCSISNIETVIEFQQKNNRLL
 ITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSI
 10 IKEEVLAYVYVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNNKQE
 GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHHHHHHHH

F11: RSV B1, wt ectodomain (SEQ ID NO: 14)

15 MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKETKCNGTDTKVLIKQELDKYKNAVTELQLLMQNTPAANNRARRREAPQYMN
 YTINTTKNLNVSISKRRKRRLGFLGFGVGSASGIAVSKVLHLEGEVNNIKNALLSTN
 KAVVSLSNGVSVLTSTKVLDLKNNYINNQLLPVNNQSCSISNIETVIEFQQKNSRLLEIN
 20 REFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIK
 EEVLAYVYVQLPIYGVITPCWKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSF
 FPQADTCKVQSNRVFCDTMNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSL
 GAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNNKLEGKN
 LYVKGEPIINYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELLHHHHHHHHH

25

F47: RSV A2, linker stabilized, IZ(S) (SEQ ID NO: 15)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKNKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSASGVAVSKVLHLEGEVNNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLK
 30 NYIDKQLLPVNNKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIKKEEVLAYVYVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN

RGIKTFSNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSD
EFDASISQVEKKIEAIEKKIEAIEKKIEAGGIEGRHHHHHHH

F47-: RSV A2, linker stabilized, IZ(S) (SEQ ID NO: 16)

5 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKNKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
FLLGVGSAIASGVAVSKVLHLEGEV NKIKSALLSTNKAVVSLSNGVSVLT SKVLDLK
NYIDKQLLPV NKQSCSISNIETVIEFQQKNNRLL EITREFSVNAGVTT P VSTYMLTNS E
LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV LAYVVQLPLYGVIDTPCW
10 KLHTSPLCTTNTKEG SNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
RGIKTFSNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSD
EFDASISQVEKKIEAIEKKIEAIEKKIEAGG

F43: RSV B1, linker stabilized, IZ(S) (SEQ ID NO: 17)

15 MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCNGTDTKV KLIKQELDKYKNAVTELQLLMQNTPAANNQARGSGSGRSL
GFLLGVGSAIASGIAVSKVLHLEGEV NKIKNALLSTNKAVVSLSNGVSVLT SKVLDL
KNYINNQLLPV NQQSCRISNIETVIEFQQKNSRLL EINREFSVNAGVTT PLSTYMLTNS
20 ELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEV LAYVVQLPIYGVIDTPCW
KLHTSPLCTTNIKEG SNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMN
SLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGI
IKTFSNGCDYVSNKGVDTVSVGNTLYYVVKLEGKNLYVKGEPIINYYDPLVFPSDEF
DASISQVEKKIEAIEKKIEAIEKKIEAGGIEGRHHHHHHH

25

F24: RSV B1, linker stabilized, fibritin (SEQ ID NO: 18)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCNGTDTKV KLIKQELDKYKNAVTELQLLMQNTPAANNQARGSGSGRSL
30 GFLLGVGSAIASGIAVSKVLHLEGEV NKIKNALLSTNKAVVSLSNGVSVLT SKVLDL
KNYINNQLLPV NQQSCRISNIETVIEFQQKNSRLL EINREFSVNAGVTT PLSTYMLTNS
ELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEV LAYVVQLPIYGVIDTPCW
KLHTSPLCTTNIKEG SNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMN
SLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGI

IKTFSNGCDYVSNKGVDTVSVGNTLYYVKNLEGKNLYVKGEPIINYYDPLVFPSDEF
 DASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFLGGI
 EGRHHHHHH

5 **A2_F24: RSV A2, linker stabilized, fibrin (SEQ ID NO: 19)**

MELLILKANAITTILTA VTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKNKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEV NKIKSALLSTNKAVVSLSNGVSVLT SKVLDLK
 NYIDKQLLPIV NKQSCSISNIETVIEFQQKNNRLL EITREFSVNAGVTT P VSTYMLTNS E
 10 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV LAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEG SNICLTRTD RGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIK TFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFLG
 15 GIEGR

F24-: RSV B1, linker stabilized, fibrin (SEQ ID NO: 20)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKETKCNGTDTKV KLIKQELDKYKNAVTELQLLMQNTPAANNQARGSGSGRSL
 20 GFL LGV GSAIASGIAVSKVLHLEGEV NKIKNALLSTNKAVVSLSNGVSVLT SKVLDL
 KNYINNQLLPIV NQQSCRISNIETVIEFQQKNSRLL EINREFSVNAGVTT PLSTYMLTNS
 ELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV LAYVVQLPIYGVIDTPCW
 KLHTSPLCTTNIKEG SNICLTRTD RGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMN
 SLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGI
 25 IK TFSNGCDYVSNKGVDTVSVGNTLYYVKNLEGKNLYVKGEPIINYYDPLVFPSDEF
 DASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFLGGI
 EGR

A2_F24 N67I+S215P: A2, linker stabilized, fibrin (SEQ ID NO: 21)

30 MELLILKANAITTILTA VTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEV NKIKSALLSTNKAVVSLSNGVSVLT SKVLDLK
 NYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL EITREFSVNAGVTT P VSTYMLTNS E
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV LAYVVQLPLYGVIDTPCW

KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFNSNGCDYVSNKGVDTVSVGNTRYVYVKNQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 5 GIEGR

F24-N67I+S215P: RSV B1, linker stabilized, fibrin (SEQ ID NO: 22)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKEIKCNGTDTKVLIKQELDKYKNAVTELQLLMQNTPAANNQARGSGSGRSLG
 10 FLLGVGSAIASGIAVSKVLHLEGEVKNKIKNALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYINNQLLPVNNQQSCRIPNIETVIEFQQKNSRLLEINREFSVNAGVTTPLSTYMLTNSE
 LLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVYVQLPIYGVIDTPCWK
 LHTSPLCTTNIKEGSNICLTRTRDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMNS
 LTLTPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGII
 15 KTFSNGCDYVSNKGVDTVSVGNTRYVYVKNLEGKNLYVKGEPIINYDPLVFPSDEFD
 ASISQVNEKINQSLAFIRRSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLGGIE
 GR

A2_F24 N67I+E92D: RSV A2, linker stabilized, fibrin (SEQ ID NO: 23)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPVNNKQSCSISNIETVIEFQQKNNRLLEITREFSVNAGVTTVPSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVYVQLPLYGVIDTPCW
 25 KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFNSNGCDYVSNKGVDTVSVGNTRYVYVKNQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

30

F24- N67I+E92D RSV B1, linker stabilized, fibrin (SEQ ID NO: 24)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKEIKCNGTDTKVLIKQELDKYKNAVTDLQLLMQNTPAANNQARGSGSGRSL
 GFLLGVGSAIASGIAVSKVLHLEGEVKNKIKNALLSTNKAVVSLSNGVSVLTISKVLDL

KNYINNQLLPVNNQQSCRISNIETVIEFQQKNSRLLEINREFSVNAGVTTPLSTYMLTNS
 ELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVVLPIYGVIDTPCW
 KLHTSPLCTTNIKEGSGNICLTRTRDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMN
 SLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGI
 5 IKTFSNGCDYVSNKGVDTVSVGNTLYYVKNLEGKNLYVKGEPIINYYDPLVFPSDEF
 DASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGGEWVLLSTFLGGI
 EGR

10 **A2_F24 N67I+K465Q RSV A2, linker stabilized, fibrin (SEQ ID NO: 25)**

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSKVLDLK
 NYIDKQLLPVNNKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTTPVSTYMLTNSE
 15 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSGNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGQSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGGEWVLLSTFLG
 20 GIEGR

F24- N67I+K465Q RSV B1, linker stabilized, fibrin (SEQ ID NO: 26)

MELLIHRLSAIFLTLAINALYTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKEIKCNGTDTKVLIKQELDKYKNAVTELQLLMQNTPAANNQARGSGSGRSLG
 25 FLLGVGSAIASGIAVSKVLHLEGEVKNIKNALLSTNKAVVSLSNGVSVLTSKVLDLK
 NYINNQLLPVNNQQSCRIPNIETVIEFQQKNSRLLEINREFSVNAGVTTPLSTYMLTNSE
 LLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVVLPIYGVIDTPCWK
 LHTSPLCTTNIKEGSGNICLTRTRDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMNS
 LTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGII
 30 KTFSNGCDYVSNKGVDTVSVGNTLYYVKNLEGQNLYVKGEPIINYYDPLVFPSDEFD
 ASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGGEWVLLSTFLGGIE
 GR

A2_F24 N67I+S46G RSV A2, linker stabilized, fibrin (SEQ ID NO: 27)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLGALRTGWYTSVITI
 ELSNIKKIKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNQARGSGSGRSL
 GFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSTKVLDL
 5 KNYIDKQLLPVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTN
 SELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPC
 WKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDT
 MNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNK
 NRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVНКQEGKSLYVKGEPIINFYDPLVFPS
 10 DEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFL
 GGIEGR

F24- N67I+S46G RSV B1, linker stabilized, fibrin (SEQ ID NO: 28)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFGALRTGWYTSVITIE
 15 LSNIKEIKCNGTDTKVLIKQELDKYKNAVTDLQLLMQNTPAANNQARGSGSGRSL
 GFLLGVGSAIASGIAVSKVLHLEGEVNKIKNALLSTNKAVVSLSNGVSVLTSTKVLDL
 KNYINNQLLPVINKQSCRISNIETVIEFQQKNSRLLEINREFSVNAGVTTPVSTYMLTNS
 ELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVVQLPIYGVIDTPCW
 KLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDTMN
 20 SLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGI
 IKTFSNGCDYVSNKGVDTVSVGNTLYYVНКLEGKNLYVKGEPIINYDPLVFPSDEF
 DASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFLGGI
 EGR

25 A2_F24 E92D+S215P: A2, linker stabilized, fibrin (SEQ ID NO: 29)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKNKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNQARGSGSGRSL
 GFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTSTKVLDL
 KNYIDKQLLPVINKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTN
 30 SELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPC
 WKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDT
 MNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNK
 NRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVНКQEGKSLYVKGEPIINFYDPLVFPS

DEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFL
GGIEGR

F24-E92D+S215P: RSV B1, linker stabilized, fibrin (SEQ ID NO: 30)

5 MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
LSNIKETKCN¹GTDTKV²KL³IKQELDKYKNAV⁴TDLQLLMQNTPAANNQARGSGSGRSL
GFL⁵GVGSAIASGIAVSKVLHLEGEVNKIKNALLSTNKAVVSLSNGVSVLT⁶SKVLDL
KNYINNQLLPIVNQ⁷QSCRIP⁸NIETVIEFQQKNSRLLEINREFSVNAGVTTPLSTYMLTN
SELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYV⁹VQ¹⁰LPIYGVIDTPC
10 WKLHTSPLCTTNIKEGSNICLTRTDRGWYCDNAGSVSFFPQADTCKVQSNRVFCDT
MNSLTLPSEVSLCNTDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKN
RGI¹¹KTFSNGCDYVSNKGVDTVSVGN¹²TLYYV¹³NKLEGKNLYVKGEPIINYYDPLVFPS
DEFDASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFL
GGIEGR

15

A2_F24 N67I+S215P+K508E: A2, linker stabilized, fibrin (SEQ ID NO: 31)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCN¹GTDAKIK²LIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
20 FLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLT³SKVLDLK
NYIDKQLLPIVNKQSCSIP⁴NIETVIEFQQKNNRLL⁵ETREFSVNAGVTT⁶VPSTYMLTNSE
LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV⁷VQ⁸LPYGV⁹IDTPCW
KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
25 RGI¹⁰KTFSNGCDYVSNKGVDTVSVGN¹¹TLYYV¹²NKQEGKSLYVKGEPIINFYDPLVFPSD
EFDASISQVNEKINQSLAFIRE-
SDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLGGIEGR

A2_F24 N67I+S215P+E487I: A2, linker stabilized, fibrin (SEQ ID NO: 32)

30 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCN¹GTDAKIK²LIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
FLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLT³SKVLDLK
NYIDKQLLPIVNKQSCSIP⁴NIETVIEFQQKNNRLL⁵ETREFSVNAGVTT⁶VPSTYMLTNSE
LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV⁷VQ⁸PLYGV⁹IDTPCW

KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFSTNGCDYVSNGVDTVSVGNTRYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 IFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 5 GIEGR

A2_F24 N67I+S215P+E487Q: A2, linker stabilized, fibrin (SEQ ID NO: 33)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 10 FLLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLK
 NYIDKQLLPIVKNQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVYVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 15 RGIKTFSTNGCDYVSNGVDTVSVGNTRYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 QFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFL
 GGIEGR

A2_F24 N67I+S215P+E487N: A2, linker stabilized, fibrin (SEQ ID NO: 34)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLK
 NYIDKQLLPIVKNQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVYVQLPLYGVIDTPCW
 25 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFSTNGCDYVSNGVDTVSVGNTRYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 NFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFL
 GGIEGR

30

A2_F24 N67I+S215P+D486N: A2, linker stabilized, fibrin (SEQ ID NO: 35)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVKNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLK

NYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL EITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV VQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 5 RGIKTF SN GCDYVS NKGV DTVSVGN TLYYV NKQEGKSLYVKGEPIINFYDPLVFPSN
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFLG
 GIEGR

A2_F24 N67I+S215P+K465E: A2, linker stabilized, fibrin (SEQ ID NO: 36)

10 MELLILKANAITTILTA VTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEV NKIKSALLSTNKAVVSLSNGVSVLT SKVLDLK
 NYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL EITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV VQLPLYGVIDTPCW
 15 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTF SN GCDYVS NKGV DTVSVGN TLYYV NKQEGESLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFLG
 GIEGR

20

A2_F24 N67I+S215P+K465Q: A2, linker stabilized, fibrin (SEQ ID NO: 37)

MELLILKANAITTILTA VTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEV NKIKSALLSTNKAVVSLSNGVSVLT SKVLDLK
 25 NYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL EITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV VQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTF SN GCDYVS NKGV DTVSVGN TLYYV NKQEGQSLYVKGEPIINFYDPLVFPSD
 30 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDG EWVLLSTFLG
 GIEGR

A2_F24 N67I+S215P+N426S: A2, linker stabilized, fibrin (SEQ ID NO: 38)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 5 NYIDKQLLPVNVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASSKNR
 GIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPSDE
 10 FDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

A2_F24 N67I+S215P+K421N: A2, linker stabilized, fibrin (SEQ ID NO: 39)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 15 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPVNVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 20 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTNCTASNKN
 RGIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

25 A2_F24 N67I+S215P+K209Q: A2, linker stabilized, fibrin (SEQ ID NO: 40)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPVNVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTPVSTYMLTNSE
 30 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFSNGCDYVSNKGVDTVSVGNTLYYVNVKQEGKSLYVKGEPIINFYDPLVFPSD

EFDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
GIEGR

A2_F24 N67I+S215P+K201Q: A2, linker stabilized, fibrin (SEQ ID NO: 41)

5 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSN¹GVSVLT²SKVLDLK
NYIDQQLLPIV³NKQSCSIPNIETVIEFQQKNNRLL⁴ETREFSVNAGVTT⁵PVSTYMLTNSE
LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV⁶LAYV⁷VQLPLYGVIDTPCW
10 KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
RGIHKTFSNGCDYVSNKGVDTVSVGNTLYYV⁸NKQEGKSLYVKGEPIINFYDPLVFPSD
EFDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
GIEGR

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A2_F24 N67I+S215P+V185N: A2, linker stabilized, fibrin (SEQ ID NO: 42)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSN¹GVSVLT²SKVLDLK
20 NYIDKQLLPIV³NKQSCSIPNIETVIEFQQKNNRLL⁴ETREFSVNAGVTT⁵PVSTYMLTNSE
LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV⁶LAYV⁷VQLPLYGVIDTPCW
KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
RGIHKTFSNGCDYVSNKGVDTVSVGNTLYYV⁸NKQEGKSLYVKGEPIINFYDPLVFPSD
25 EFDASISQVNEKINQSLAFIRKSDELLESAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
GIEGR

A2_F24 N67I+S215P+G184N: A2, linker stabilized, fibrin (SEQ ID NO: 43)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
30 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSN¹GVSVLT²SKVLDLK
NYIDKQLLPIV³NKQSCSIPNIETVIEFQQKNNRLL⁴ETREFSVNAGVTT⁵PVSTYMLTNSE
LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEV⁶LAYV⁷VQLPLYGVIDTPCW
KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM

NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFNSNGCDYVSNGVDTVSVGNTRYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

5

A2_F24 N67I+S215P+N175P: A2, linker stabilized, fibrin (SEQ ID NO: 44)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTPKAVVSLSNGVSVLTISKVLDLK
 10 NYIDKQLLPIVVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVYVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFNSNGCDYVSNGVDTVSVGNTRYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 15 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

A2_F24 N67I+S215P+E92D: A2, linker stabilized, fibrin (SEQ ID NO: 45)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 20 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPIVVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVYVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 25 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFNSNGCDYVSNGVDTVSVGNTRYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

30 A2_F24 N67I+S215P+K80E: A2, linker stabilized, fibrin (SEQ ID NO: 46)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIEQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLGF
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTISKVLDLKN
 YIDKQLLPIVVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSEL

LSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWK
 LHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNS
 LTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGI
 IKTFSNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSDEF
 5 DASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGGEWVLLSTFLGGI
 EGR

A2_F24 N67I+S215P+K77E: A2, linker stabilized, fibrin (SEQ ID NO: 47)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 10 LSNIKKIKCNGTDAKIELIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLGF
 LLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTISKVLDLKN
 YIDKQLLPIVVKQSCSIPIETVIEFQQKNNRLEITREFSVNAGVTTPVSTYMLTNSEL
 LSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCWK
 LHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNS
 15 LTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGI
 IKTFSNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSDEF
 DASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGGEWVLLSTFLGGI
 EGR

20 A2_F24 N67I+S215P+S46G: A2, linker stabilized, fibrin (SEQ ID NO: 48)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLGALRTGWYTSVITI
 ELSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSL
 GFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTISKVLDL
 KNYIDKQLLPIVVKQSCSIPIETVIEFQQKNNRLEITREFSVNAGVTTPVSTYMLTN
 25 SELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPC
 WKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDT
 MNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNK
 NRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPS
 DEFDAISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGGEWVLLSTFL
 30 GGIEGR

A2_F24: RSV S46G A2, linker stabilized, fibrin (SEQ ID NO: 49)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLGALRTGWYTSVITI
 ELSNIKKKNCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSL
 GFLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDL
 5 KNYIDKQLLPVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTN
 SELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPC
 WKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDT
 MNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNK
 NRGIIKTFSNGCDYVSNGKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPS
 10 DEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFL
 GGIEGR

A2_F24: RSV K465Q A2, linker stabilized, fibrin (SEQ ID NO: 50)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 15 LSNIKKKNKNCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 20 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFSNGCDYVSNGKGVDTVSVGNTLYYVVKQEGQSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
 GIEGR

25 A2_F24: RSV N67I A2, linker stabilized, fibrin (SEQ ID NO: 51)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPVINKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTNSE
 30 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTFSNGCDYVSNGKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSD

EFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFLG
GIEGR

A2_F24: RSV E92D A2, linker stabilized, fibrin (SEQ ID NO: 52)

5 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKKNCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNQARGSGSGRSL
GFLLGVGSAIASGVAVSKVLHLEGEVNNIKSALLSTNKAVVSLSNGVSVLTSKVLDL
KNYIDKQLLPIVNBKQSCSISNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTN
SELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPC
10 WKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDT
MNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNK
NRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNBKQEGKSLYVKGEPIINFYDPLVFPS
DEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGWVLLSTFL
GGIEGR

15

RSV F protein CL57-v224 full length sequence (SEQ ID NO: 69)

MELPILKTNAITTILAAVTLCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKENKNCNGTDAKVLIKQELDKYKNAVTELQLLMQSTPAANNRARRRELPRFMN
YTLNNTKNNNVTLSSKKRKRRLGFLFGVGSAGIAVSKVLHLEGEVNNIKSALLST
20 NKAVVSLSNGVSVLTSKVLDLKNYIDKQLLPIVNBKQSCSISNIETVIEFQQKNNRLLLEI
TREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNIDIFNPKYDCKIMTSKTDVSSSVI
TSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNBKQEG
25 KSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNVGKSTT
NIMITTIIIVIIIVILLLLIAVGLFLYCKARSTPVTLSKDQLSGINNIAFSN

Ectodomain, RSV CL57-v224 (SEQ ID NO: 70)

MELPILKTNAITTILAAVTLCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKENKCNGTDAKVLIKQELDKYKNAVTELQLLMQSTPAANNRARRRELPRFMN
 YTLNNTKNNNVTLSSKKRKRRLGFLGVGSAIASGIAVSKVLHLEGEVNIKSALLST
 5 NKAUVSLSNGVSVLTSSKVLDLKNIYDKQLLPVNVKQSCSISNIETVIEFQQKNNRLLEI
 TREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNIDIFNPKYDCKIMTSKTDVSSSVI
 TSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNVKQEG
 10 KSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELL

PreF, RSV A2, fibrin (SEQ ID NO: 71)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKNCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRRELPRFMN
 15 YTLNNAKKTNTVTLSSKKRKRRLGFLGVGSAIASGVAVSKVLHLEGEVNIKSALLS
 TNKAVVLSNGVSVLTSSKVLDLKNIYDKQLLPVNVKQSCSISNIETVIEFQQKNNRLLEI
 ITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSI
 IKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 20 ITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNVKQE
 GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAP
 RDGQAYVRKDGGEWVLLSTFL

PreF N67I S215P, RSV A2, fibrin (SEQ ID NO: 72)

25 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRRELPRFMNY
 TLNNAKKTNTVTLSSKKRKRRLGFLGVGSAIASGVAVSKVLHLEGEVNIKSALLST
 NKAUVSLSNGVSVLTSSKVLDLKNIYDKQLLPVNVKQSCSIPNIETVIEFQQKNNRLLEI
 TREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 30 KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYVNVKQE
 GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAP
 RDGQAYVRKDGGEWVLLSTFL

PreF N67I S215P, RSV B1, fibrin (SEQ ID NO: 73)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKEIKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNRARRREAPQYMN
 5 YTINTTKNLNVSISKRRRFLGFLGVSASGIAVSKVLHLEGEVNIKINALLSTN
 KAVVSLSNGVSVLTSTKVLDLKNYINNQLLPIVNQQSCRIPNIETVIEFQQKNSRLLEIN
 REFSVNAGVTTPLSTYMLTNSELLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIK
 EEVLAYVVQLPIYGVIDTPCWKLHTSPLCTTNIKEGSNICLTRTRDRGWYCDNAGSVSF
 10 GAIVSCYGKTKCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTLYYVKNLEGKN
 LYVKGEPIINYDPLVFPSDEFDASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRD
 GQAYVRKDGWVLLSTFL

RSV N67I S215P, RSV CL57-v224, fibrin (SEQ ID NO: 74)

15 MELPILKTNAITTILAAVTLCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKEIKCNGTDAKVKLIKQELDKYKNAVTELQLLMQSTPAANNRARRRELPRFMN
 YTLNNTKNNNVTLSSKKRRRFLGFLGVSASGIAVSKVLHLEGEVNIKISALLST
 NKAVVSLSNGVSVLTSTKVLDLKNYIDKQLLPIVNKQSCSIPNIETVIEFQQKNNRLLEI
 TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 20 KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNIDIFNPKYDCKIMTSKTDVSSSVI
 TSLGAIVSCYGKTKCTASNKNRGIKTFSSNGCDYVSNKGVDTVSVGNTLYYVKNQEG
 KSLEYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPR
 DGQAYVRKDGWVLLSTFL

25

PreFL N67I S215P, RSV B1, fibrin, Loop (SEQ ID NO: 22)

MELLIHRLSAIFLTLAINALYLTSSQNITEEFYQSTCSAVSRGYFSALRTGWYTSVITIE
 LSNIKEIKCNGTDTKVKLIKQELDKYKNAVTELQLLMQNTPAANNQARGSGSGRSLG
 FLLGVGSAIASGIAVSKVLHLEGEVNIKINALLSTNKAVVSLSNGVSVLTSTKVLDLK
 30 NYINNQLLPIVNQQSCRIPNIETVIEFQQKNSRLLEINREFSVNAGVTTPLSTYMLTNSE
 LLSLINDMPITNDQKKLMSSNVQIVRQQSYSIMSIIKEEVLAYVVQLPIYGVIDTPCWK
 LHTSPLCTTNIKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNS
 LTLPSEVSLCNDIFNSKYDCKIMTSKTDISSSVITSLGAIVSCYGKTKCTASNKNRGI

KTFSNGCDYVSNKGVDTVSVGNTLYYVKNLEGKNLYVKGEPIINYDPLVFPSDEFD
ASISQVNEKINQSLAFIRRSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFL

PreFL N67I S215P, RSV CL57-v224, fibritin, Loop (SEQ ID NO: 75)

5 MELPILKTNAITTILAAVTLCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKEIKCNGTDAKVLIKQELDKYKNAVTELQLLMQSTPAANNQARGSGSGRSLG
FLLGVGSAIASGIAVSKVLHLEGEVNNIKSALLSTNKAVVSLSNGVSVLTSTKVLDLKN
YIDKQLLPVNVKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTTPVSTYMLTNSEL
LSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVYVQLPLYGVIDTPCWK
10 LHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTMNS
LTLPSEVNLCNIDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKNRGII
KTFSNGCDYVSNKGVDTVSVGNTLYYVKNQEGKSLYVKGEPIINFYDPLVFPSDEFD
ASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAPRDGQAYVRKDGEWVLLSTFL

15 PreF N67I S215P E487Q, RSV A2, fibritin (SEQ ID NO: 76)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNY
TLNNAKKTNTVTLSSKKRKRRLGFLGVGSAIASGVAVSKVLHLEGEVNNIKSALLST
NKAVVSLSNGVSVLTSTKVLDLKNYIDKQLLPVNVKQSCSIPNIETVIEFQQKNNRLLLEI
20 TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
KEEVLAYVYVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
ITSLGAIVSCYGKTKCTASNKNRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVKNQGE
GKSLYVKGEPIINFYDPLVFPSDQFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEA
25 PRDGQAYVRKDGEWVLLSTFL

PreF N67I S215P K201N, RSV A2, fibritin (SEQ ID NO: 77)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNY
30 TLNNAKKTNTVTLSSKKRKRRLGFLGVGSAIASGVAVSKVLHLEGEVNNIKSALLST
NKAVVSLSNGVSVLTSTKVLDLKNYIDNQLLPVNVKQSCSIPNIETVIEFQQKNNRLLLEI
TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
KEEVLAYVYVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV

ITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYV NKQE
GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAP
RDGQAYVRKDGGEWVLLSTFL

5 **PreF N67I S215P E92D, RSV A2, fibritin (SEQ ID NO:78)**

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNRARRELPRFMNY
TLNNAKKTNTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLST
NKAVVSLSNGVSVLTSTKVLDLKNIYDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLLEI
10 TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
ITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYV NKQE
GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAP
15 RDGQAYVRKDGGEWVLLSTFL

PreF N67I S215P D486N, RSV A2, fibritin (SEQ ID NO: 79)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNY
20 TLNNAKKTNTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLST
NKAVVSLSNGVSVLTSTKVLDLKNIYDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLLEI
TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
25 ITSLGAIVSCYGKTKCTASNKNRGIKTFSGCDYVSNKGVDTVSVGNTLYYV NKQE
GKSLYVKGEPIINFYDPLVFPSNEFDASISQVNEKINQSLAFIRKSDELLSAIGGYIPEAP
RDGQAYVRKDGGEWVLLSTFL

Fwt N67I S215P, membrane-bound RSV F, A2, (SEQ ID NO: 80)

30 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNY
TLNNAKKTNTLSKKRKRRFLGFLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLST
NKAVVSLSNGVSVLTSTKVLDLKNIYDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLLEI
TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII

KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVVKQE
 GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
 5 TNIMITTHIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

Fsl N67I S215P, membrane-bound RSV F, A2, (SEQ ID NO: 81)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 10 FLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTISKVLDLK
 NYIDKQLLPIVNKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 15 RGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVVKQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLHNVNAVKSTTNIMITTHIIVILLSLIAVGLLL
 YCKARSTPVTLSKDQLSGINNIAFSN

Fwt N67I S215P E92D, membrane-bound RSV F, A2, (SEQ ID NO: 82)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNRARRELPRFMNY
 TLNNAKKTNTLSKKRKRRLGFLGVGSAIASGVAVSKVLHLEGEVNKIKSALLST
 NKAVVSLSNGVSVLTISKVLDLKNYIDKQLLPIVNKQSCSIPNIETVIEFQQKNNRLLLEI
 TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 25 KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTFSTNGCDYVSNKGVDTVSVGNTLYYVVKQE
 GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
 TNIMITTHIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

30

Fsl N67I S215P E92D, membrane-bound RSV F, A2, (SEQ ID NO: 83)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTDLQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLTISKVLDLK

NYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL EITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV VQQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 5 RGIKTF SNGCDYVS NKGVDTVSVGNTLYYV NKQEGKSLYVKGEPIINFYDPLVFPSD
 EFDASISQVNEKINQSLAFIRKSDELLHNVNAVKSTTNIMITTHIIVII VILLSLIAVG LLL
 YCKARSTPVTLSKDQLSGINNIAFSN

Fwt N67I S215P E487Q, membrane-bound RSV F, A2, (SEQ ID NO: 84)

10 MELLILKANAITTILTA VTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARELPRFMNY
 TLNNAKKTNTVLSKKRKRRLGFL LGVGSIAIASGVAVSKVLHLEGEVNKIKSALLST
 NKAVVSLSNGVSVLT SKVLDLKNYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL E
 TREFSVNAGVTTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 15 KEEVLAYV VQQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTF SNGCDYVS NKGVDTVSVGNTLYYV NKQE
 GKSLYVKGEPIINFYDPLVFPSDQFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
 TNIMITTHIIVII VILLSLIAVG LLLYCKARSTPVTLSKDQLSGINNIAFSN

20

Fsl N67I S215P E487Q, membrane-bound RSV F, A2, (SEQ ID NO: 85)

MELLILKANAITTILTA VTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSIAIASGVAVSKVLHLEGEVNKIKSALLSTNKAVVSLSNGVSVLT SKVLDLK
 25 NYIDKQLLPIV NKQSCSIPNIETVIEFQQKNNRLL EITREFSVNAGVTTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYV VQQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTRDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTF SNGCDYVS NKGVDTVSVGNTLYYV NKQEGKSLYVKGEPIINFYDPLVFPSD
 30 QFDASISQVNEKINQSLAFIRKSDELLHNVNAVKSTTNIMITTHIIVII VILLSLIAVG LLL
 YCKARSTPVTLSKDQLSGINNIAFSN

Fwt N67I S215P D486N, membrane-bound RSV F, A2, (SEQ ID NO: 86)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMNY
 TLNNAKKTNTVTLSSKKRKRRLGFLGFGVSAIASGVAVSKVLHLEGEVNNIKSALLST
 5 NKA VVSLSNGVSVLT SKVLDLKNYIDKQLLPV NKQSCSIPNIETVIEFQQKNNRLLEI
 TREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 KEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTF SNGCDYVS NKGVDTVSVGNTLYYV NKQE
 10 GKSLYVKGEPIINFYDPLVFPSNEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
 TNIMITTHIIVILLSLIAVG LLLYCKARSTPVTLSKDQLSGINNIAFSN

Fsl N67I S215P D486N, membrane-bound RSV F, A2, (SEQ ID NO: 87)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITIE
 15 LSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSLG
 FLLGVGSAIASGVAVSKVLHLEGEVNNIKSALLSTNKAVVSL SNGVSVLT SKVLDLKNYIDKQLLPV NKQSCSIPNIETVIEFQQKNNRLLEITREFSVNAGVTTPVSTYMLTNSE
 LLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPCW
 KLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDTM
 20 NSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNKN
 RGIKTF SNGCDYVS NKGVDTVSVGNTLYYV NKQEGKSLYVKGEPIINFYDPLVFPSN
 EFDASISQVNEKINQSLAFIRKSDELLHNVNAVKSTTNIMITTHIIVILLSLIAVG LLL
 YCKARSTPVTLSKDQLSGINNIAFSN

25 Fwt N67I S215P S46G, membrane-bound RSV F, A2, (SEQ ID NO: 88)

MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLGALRTGWYTSVITI
 ELSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNRARRELPRFMN
 YTLNNAKKTNTVTLSSKKRKRRLGFLGFGVSAIASGVAVSKVLHLEGEVNNIKSALLS
 TNKAVVSL SNGVSVLT SKVLDLKNYIDKQLLPV NKQSCSIPNIETVIEFQQKNNRLLE
 30 ITREFSVNAGVTTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSII
 IKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGS
 VSFFPQAETCKVQSNRVFCDTMNSLTLPSEVNLCNVDIFNPKYDCKIMTSKTDVSSSV
 ITSLGAIVSCYGKTKCTASNKNRGIKTF SNGCDYVS NKGVDTVSVGNTLYYV NKQE

GKSLYVKGEPIINFYDPLVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKST
TNIMITTHIIVIIIVILLSLIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN

Fsl N67I S215P S46G, membrane-bound RSV F, A2, (SEQ ID NO: 89)

5 MELLILKANAITTILTAVTFCFASGQNITEEFYQSTCSAVSKGYLGALRTGWYTSVITI
ELSNIKKIKCNGTDAKIKLIKQELDKYKNAVTELQLLMQSTPATNNQARGSGSGRSL
GFLLGVGSAIASGVAVSKVLHLEGEVNIKISALLSTNKAVVSLSNGVSVLTSKVLDL
KNYIDKQLLPIVNBKQSCSIPNIETVIEFQQKNNRLLLEITREFSVNAGVTTPVSTYMLTN
SELLSLINDMPITNDQKKLMSNNVQIVRQQSYSIMSIIKEEVLAYVVQLPLYGVIDTPC
10 WKLHTSPLCTTNTKEGSNICLTRTDRGWYCDNAGSVSFFPQAETCKVQSNRVFCDT
MNSLTLPSEVNL CNVDIFNPKYDCKIMTSKTDVSSSVITSLGAIVSCYGKTKCTASNK
NRGIKTFSNGCDYVSNKGVDTVSVGNTLYYVNBKQEGKSLYVKGEPIINFYDPLVFPS
DEFDASISQVNEKINQSLAFIRKSDELLHNVNAVKSTTNIMITTHIIVIIIVILLSLIAVGLL
LYCKARSTPVTLSKDQLSGINNIAFSN

15

CR9501 heavy chain (SEQ ID NO: 53):

QVQLVQSGPGLVKPSQTLALTCNVSGASINSNYYWTWIRQRPGGGLEWIGHISYTG
NTYYTPSLKSRLSMSLETSQSQFSLRLTSVTAADSAVYFCAACGAYVLISNCGWFDS
WGQGTQVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPPTVSWNSGAL
20 TSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSC

CR9501 light chain (SEQ ID NO: 61):

EIVMTQSPSSLSASIGDRVTITCQASQDISTYLNWYQQKPGQAPRLLIYGASNLETGVP
SRFTGSGYGTDFSVTISSLQPEDIATYYCQQYQYLPYTFAPGKVEIKRTVAAPSVFIF
25 PPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDESTYS
LSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

CR9502 heavy chain (SEQ ID NO: 57):

EVQLLQSGAELKKPGASVKISCKTSGFTFSGHTIAWVRQAPGQGLEWMGWVSTNNG
30 NTEYAQKIQRVTMTMDTSTSTVYMELRSLTSDDTAVYFCAREWLVMGGFAFDHW
GQGTLTIVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPPTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSC

CR9502 light chain (SEQ ID NO: 65):

QSVLTQASSVSVAPGQTARITCGANNIGSQNVHWYQQKPGQAPVLVVYDDRDRPSG
IPDRFSGSNSGNTATLTISRVEAGDEADYYCQVWDSSRDQAVIFGGGTKLTVLGQPK
5 AAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQS
NNKYAASSYLSLTPEQWKSHRSYSCQVTHEGSTVEKTIAPTECS

Claims

1. A recombinant pre-fusion respiratory syncytial virus (RSV) Fusion (F) polypeptide,
comprising at least one epitope that is specific to the pre-fusion conformation F
5 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: 54, a
heavy chain CDR2 region of SEQ ID NO: 55, a heavy chain CDR3 region of SEQ ID
NO: 56 and a light chain CDR1 region of SEQ ID NO: 62, a light chain CDR2 region
of SEQ ID NO: 63, and a light chain CDR3 region of SEQ ID NO: 64 and/or a pre-
10 fusion specific monoclonal antibody, comprising a heavy chain CDR1 region of SEQ
ID NO: 58, a heavy chain CDR2 region of SEQ ID NO: 59, a heavy chain CDR3
region of SEQ ID NO: 60 and a light chain CDR1 region of SEQ ID NO: 66, a light
chain CDR2 region of SEQ ID NO: 67, and a light chain CDR3 region of SEQ ID
NO: 68.
15
2. Pre-fusion RSV F polypeptide according to claim 1, wherein the polypeptide is
trimeric.
3. Pre-fusion RSV F polypeptide according to claim 1 or 2, wherein the polypeptide
20 comprises a mutation of the amino acid residue on position 67 and/or a mutation of
the amino acid residue on position 215.
4. Pre-fusion RSV F polypeptide according to claim 1, 2 or 3, wherein the polypeptide
comprises a F1 domain and a F2 domain, and a linking sequence comprising from 1 to
25 10 amino acid residues, linking said F1 domain to said F2 domain.

5. Pre-fusion RSV F polypeptide according to any of the preceding claims 1-4, comprising a truncated F1 domain and a F2 domain, and a linking sequence comprising 1 to 10 amino acid residues, linking said F1 to said F2 domain, wherein polypeptide comprises at least one stabilizing mutation in the F1 and/or F2 domain as compared to the RSV F1 and/or F2 domain in the wild-type RSV F protein, wherein the at least one stabilizing mutation is selected from the group consisting of:
- (a) a mutation of amino acid residue N/T on position 67.
 - (b) a mutation of amino acid residue S on position 215.
6. Pre-fusion RSV F polypeptide according to any of the preceding claims 1-5, wherein the at least one stabilizing mutation is selected from the group consisting of:
- (a) a mutation of amino acid residue N/T on position 67 into I; and
 - (b) a mutation of amino acid residue S on position 215 into P.
7. Pre-fusion RSV F polypeptide according to claim 5 or 6, wherein the polypeptide comprises a heterologous trimerization domain linked to said truncated F1 domain.
8. Pre-fusion RSV F polypeptide according to any one of the claims 1-7, wherein the polypeptide comprises at least one further mutation, wherein said mutation is selected from the group consisting of:
- (a) a mutation of the amino acid residue on position 46;
 - (b) a mutation of the amino acid residue on position 77;
 - (c) a mutation of the amino acid residue on position 80;
 - (d) a mutation of the amino acid residue on position 92;
 - (e) a mutation of the amino acid residue on position 175;

- (f) a mutation of the amino acid residue on position 184;
 - (g) a mutation of the amino acid residue on position 185;
 - (h) a mutation of the amino acid residue on position 201;
 - (i) a mutation of the amino acid residue on position 209;
 - 5 (j) a mutation of the amino acid residue on position 421;
 - (k) a mutation of the amino acid residue on position 426;
 - (l) a mutation of the amino acid residue on position 465;
 - (m) a mutation of the amino acid residue on position 486;
 - (n) a mutation of the amino acid residue on position 487; and
 - 10 (o) a mutation of the amino acid residue on position 508.
9. Pre-fusion RSV F polypeptide according to claim 8, wherein the at least one further mutation is selected from the group consisting of:
- (a) a mutation of the amino acid residue S on position 46 into G;
 - 15 (b) a mutation of the amino acid residue K on position 77 into E;
 - (c) a mutation of the amino acid residue K on position 80 into E;
 - (d) a mutation of the amino acid residue E on position 92 into D;
 - (e) a mutation of the amino acid residue N on position 175 into P;
 - (f) a mutation of the amino acid residue G on position 184 into N;
 - 20 (g) a mutation of the amino acid residue V on position 185 into N;
 - (h) a mutation of the amino acid residue K on position 201 into Q;
 - (i) a mutation of the amino acid residue K on position 209 into Q;
 - (j) a mutation of the amino acid residue K on position 421 into N;
 - (k) a mutation of the amino acid residue N on position 426 into S;
 - 25 (l) a mutation of the amino acid residue K on position 465 into E or Q;

- (m) a mutation of the amino acid residue D on position 486 into N;
- (n) a mutation of the amino acid residue E on position 487 into Q, N or I; and
- (o) a mutation of the amino acid residue K on position 508 into E.

5 10. Pre-fusion RSV F polypeptide according to any of the preceding claims 1-9, wherein
the polypeptide comprises at least two mutations.

11. Pre-fusion RSV F polypeptide according to claim 10, wherein the at least two
mutations comprise a mutation of amino acid residue N/T on position 67 into I; and a
10 mutation of amino acid residue S on position 215 into P.

12. Pre-fusion RSV F polypeptide according to claim 11, wherein the polypeptide
comprises at least one further mutation selected from the group consisting of:

- (a) a mutation of the amino acid residue S on position 46 into G;
- 15 (b) a mutation of the amino acid residue K on position 77 into E;
- (c) a mutation of the amino acid residue K on position 80 into E;
- (d) a mutation of the amino acid residue E on position 92 into D;
- (e) a mutation of the amino acid residue N on position 175 into P;
- (f) a mutation of the amino acid residue G on position 184 into N;
- 20 (g) a mutation of the amino acid residue V on position 185 into N;
- (h) a mutation of the amino acid residue K on position 201 into Q;
- (i) a mutation of the amino acid residue K on position 209 into Q;
- (j) a mutation of the amino acid residue K on position 421 into N;
- (k) a mutation of the amino acid residue N on position 426 into S;
- 25 (l) a mutation of the amino acid residue K on position 465 into E or Q;

- (m) a mutation of the amino acid residue D on position 486 into N;
- (n) a mutation of the amino acid residue E on position 487 into Q, N or I; and
- (o) a mutation of the amino acid residue K on position 508 into E.

5 13. Pre-fusion RSV F polypeptide according to any one of the preceding claims 1-12,
 wherein the heterologous trimerization domain comprises the amino acid sequence
 EKKIEAIEKKIEAIEKKIEA (SEQ ID NO: 3)

 14. Pre-fusion RSV F polypeptide according to claim 13, wherein the trimerization
10 domain is linked to amino acid residue 495 of the RSV F protein.

 15. Pre-fusion RSV F polypeptide according to any one of the claims 1-14, wherein the
 heterologous trimerization domain comprises the amino acid sequence
 GYIPEAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO: 4).

15 16. Pre-fusion RSV F polypeptide according to claim 15, wherein the trimerization
 domain is linked to amino acid residue 513 of the RSV F protein.

 17. Pre-fusion RSV F polypeptide according to any one of the preceding claims, wherein
20 the linker between the F1 and the F2 domain comprises 5 amino acid residues.

 18. Pre-fusion RSV F polypeptide according to claim 17, wherein the linker comprises
 the amino acid sequence GSGSG (SEQ ID NO: 5).

19. Pre-fusion RSV F polypeptide according to any of the preceding claims, wherein the F1 domain and/or the F2 domain are from an RSV A strain.

20. Pre-fusion RSV F polypeptide according to any of the preceding claims, wherein the F1 domain and/or the F2 domain are from an RSV B strain.

21. Pre-fusion RSV F polypeptide according to any of the preceding claims, wherein the polypeptide is stable for at least 30 minutes at 55° C, preferably at 58° C, more preferably at 60° C.

22. Pre-fusion RSV F polypeptide according to any of the preceding claims, wherein the polypeptide is stable after storage at 4°C for at least 30 days, preferably at least 60 days, preferably at least 6 months, even more preferably at least 1 year.

23. Pre-fusion RSV F polypeptide according to any one of the preceding claims, wherein the polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 21 – SEQ ID NO: 52 and 71-89.

24. Pre-fusion RSV F polypeptide according to any of the preceding claims, wherein the polypeptide does not comprise a HIS-Tag.

25. Nucleic acid molecule encoding a pre-fusion RSV F polypeptide according to any one of the preceding claims 1-24.

26. Nucleic acid molecule according to claim 25, wherein the nucleic acid molecule has been codon-optimized for expression in mammalian cells.

27. Vector comprising a nucleic acid molecule according to claim 25 or claim 26.

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28. Composition comprising a pre-fusion RSV F polypeptide according to any of the claims 1-24, a nucleic acid molecule according to claim 25 or claim 26 and/or a vector according to claim 27.

10 29. Pre-fusion RSV F polypeptide according to any of the claims 1-24, a nucleic acid molecule according to claim 25 or claim 26 and/or a vector according to claim 27 for use in inducing an immune response against RSV F protein.

15 30. Pre-fusion RSV F polypeptide according to any of the claims 1-24, a nucleic acid molecule according to claim 25 or claim 26 and/or a vector according to claim 27 for use as a vaccine.

20 31. Pre-fusion RSV F polypeptide according to any of the claims 1-24, a nucleic acid molecule according to claim 25 or claim 26 and/or a vector according to claim 27 for use in the prophylaxis and/or treatment of RSV infection.

25 32. A method for stabilizing the pre-fusion conformation of an RSV F polypeptide, comprising introducing one or more mutations in a RSV F1 and/or F2 domain, as compared to the wild-type RSV F1 and/or F2 domain, wherein the one or more mutations are selected from the group consisting of:

- (e) a stabilizing mutation in a region that locks the HRA domain from hinging adjacent to the conserved 69-212 disulfide bridge, said region comprising the amino acid residues 66-68 and 214-216,
- 5 (f) a mutation in the helix comprising the amino acid residues 76-98 at the C-terminus of the F2 domain;
- (g) a mutation that reduces the negative charge repulsion between the top of the HRB stem region comprising amino acids 486, 487; and
- (h) a stabilizing mutation in the HRA region.

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33. Method according to claim 32, wherein the mutation in the HRA hinge region is at position 67.

34. Method according to claim 32 or 33, wherein the mutation in the HRA hinge region is at position 215.

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35. Method according to claim 32, 33 or 34, wherein the mutation in the HRA hinge region is at position 66 or 68, and/or at position 214 or 216.

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36. Method according to claim 32, wherein the mutation in the helix is at position 77.

37. Method according to claim 32, wherein the mutation in the helix is at position 80.

38. Method according to claim 36 or 37, wherein the amino acid residue at position 77

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and/or 80 is changed into a negatively charged amino acid.

39. Method according to any one of the claims 32-38, wherein the mutation is at position 92.

5 40. Method according to claim 32, wherein the mutation that reduces the negative charge repulsion between the top of the HRB stem region comprising amino acids 486, 487, 489.

41. Method according to claim 40, wherein the mutation is at position 489.

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42. Method according to claim 40, wherein the mutation is at position 486.

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43. Method according to claim 32, wherein the mutation stabilizes the beta-turns between the amino acid residues 175-193.

44. Method according to claim 43, wherein the mutation is stabilizing the turn at position 175.

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45. Method according to claim 43, wherein the mutation is stabilizing the turn at position 184 -185.

46. Stabilized pre-fusion RSV F polypeptide, obtainable by a method according to any one of the claims 32-45.

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